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13. ABSTRACT <i>(Maximum 200 words)</i> <p>Objective: To draw together in a common environment doctoral students from different basic science disciplines and provide them with a strong multidisciplinary background in breast cancer research.</p> <p>Approach: 1) Provide future basic scientists (predoctoral students) in a given discipline (e.g., physiology, biochemistry, etc.) a stable multidisciplinary foundation from which to develop cutting-edge research in breast diseases, resulting in a dissertation dealing with a specific breast cancer research topic. 2) From these doctoral (Ph.D.) students, develop a cadre of postdoctoral fellows who will devote their entire professional careers to the field of breast cancer research and will be able to generate their own funding through competitive grants and contracts. It is expected that this group of researchers will swiftly and effectively provide translational (i.e., clinically relevant) bench research results into the clinical arena.</p>				
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PRINCIPAL INVESTIGATOR: Tapas Das Gupta, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
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FOREWORD

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T. K. Das Gupta 9/26/97
PI - Signature Date

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5. Introduction

The purpose of this grant was to draw together in a common environment doctoral students from different basic science disciplines and provide them with a strong multidisciplinary background in breast cancer research.

We tried to provide the milieu of a multidisciplinary basic research forum for training the future generation of basic scientists so that they could develop an appropriate basis from which to pursue clinically relevant (i.e., translational) research in breast cancer. We also tried to provide a stable multidisciplinary foundation from which to develop cutting-edge research in breast diseases, resulting in a dissertation dealing with a specific breast cancer research topic.

6. Body

The tasks listed in the grant proposal were:

Task 1: Provide to predoctoral fellows in various disciplines (e.g., Physiology, Biochemistry) a multidisciplinary basic foundation and emerging knowledge in breast cancer biology.

Task 2: Develop a well-focused dissertation theme related specifically to breast cancer.

Task 3: Generate a cadre of well-trained doctoral students (Ph.D.s) who will devote their professional careers to the field of breast cancer research and will be able to generate their own funding through competitive grants and contracts.

Task 4: Provide the milieu of a multidisciplinary basic research forum for training and educating clinical scientists, so that they can develop an appropriate basis from which to pursue clinical research.

During the grant, we established a participating faculty committee for selection of the three graduate students funded by the grant. Although, in the original application, we proposed to select nine predoctoral students for the fellowship, the requested funding was reduced to support only three students. Therefore, we supported only three students with the fellowships.

The selection committee consisted of Dr. Das Gupta (chairman) and Drs. E.P. Cohen (Department of Microbiology and Immunology), R.L. Davidson (Department of Genetics), R.R. Mehta (Department of Surgical Oncology), M.B. Mokyr (Department of Biochemistry), P. Raychaudhuri (Department of Biochemistry), and I.G. Roninson (Department of Genetics). This committee selected three graduate students from candidates nominated by each basic science department to be supported by this training grant. The candidates selected were Lisa Shamon, Margaret R. Waltz, and Lavanya Lall. These three were chosen from a pool of 22 candidates from all the basic science departments. The paramount reasons for selecting these three graduate students were their commitment to breast cancer research, their grades, and, finally, the disciplines in which they were pursuing their graduate studies. Ms. Shamon was a student in the Department of Medicinal Chemistry and Pharmacognosy in the College of Pharmacology; Ms. Waltz was a student in the Department of Microbiology and Immunology, College of Medicine; and Ms. Lall is in the Department of Genetics.

These graduate students not only took all the courses necessary to fulfill the requirements of the parent department, but also completed a course offered by the program faculty as a prerequisite for the Breast Cancer

Research Fellowship. This was a specially designed course, titled "Basic Concepts in Cancer Biology." The objective of this course was to provide the predoctoral students with a larger vista in cancer biology, so that these students evolved into mature cancer biologists. The course was offered in the form of a seminar once a month (2 hours). Developing this program, we planned a 3- to 4-credit hour course. However, with three students in the program, it was very difficult to execute it as a University graduate-level course. We requested to initiate a course with the Graduate College; however, our request was turned down due to the small number of students interested at that time. Thus, the course was organized as a seminar. These seminars were mandatory for the fellows, and all faculty members (basic scientists and clinical faculty members) were expected to attend. Also, the fellows had to present their research work as a formal seminar. The workshops are on informal basis. For example, Lisa Shamon spent time in Dr. Constantinou's laboratory learning topoisomerase assays and in Dr. R.R. Mehta's laboratory learning immunocytochemistry. Miss Lall spent time in Dr. R.G. Mehta's laboratory learning carcinogen transformation of human breast epithelial HBL 1000 cells. This association not only taught the fellows other techniques, it also allowed them to be part of a publication when one was generated from this effort.

Each of these trainees also developed a research program dealing with some aspect of breast cancer, under the respective preceptorship of Drs. John M. Pezzuto (Department of Medicinal Chemistry & Pharmacognosy), Edward A. Cohen (Department of Microbiology and Immunology), and Richard L. Davidson (Department of Genetics). The trainees' dissertations are written about the research performed in breast cancer. Dr. Das Gupta was a member of the respective thesis committees of these three graduate students. Descriptions and progress reports on their research activities are provided below.

Research Projects of Lisa Shamon (Preceptor: Dr. J.M. Pezzuto)

Title: Natural Products in the Chemoprevention and Chemotherapy of Breast Cancer: Antimutagens and Inhibitors of HER-2/neu Proto-oncogene

Description

The HER-2/neu proto-oncogene encodes for a 185-kD protein with intrinsic tyrosine kinase activity. Oncogenic effects are manifested when the protein is overexpressed, occurring in approximately 25%-30% of invasive breast carcinomas and in >80% of comedo ductal carcinoma in situ (comedo-DCIS) (1). Although the mechanism is not fully understood, overexpression of HER-2/neu has been associated with aggressive disease, increased probability of tumor recurrence, and poor patient survival rates (2,3). Thus, the presence of HER-2/neu in both preinvasive and invasive disease makes this protein a suggested target for antitumor agents. In order to screen for potential inhibitors of HER-2/neu, 300 compounds were selected from an inventory of natural product isolates. In this project, several in vitro and in vivo methods were used to screen for inhibitors of HER-2/neu and to follow-up specific leads with more in-depth mechanistic studies.

To summarize, three human breast carcinoma cell lines developed in our departmental laboratory (UIISO-BCA-1, UIISO-BCA-2, and MAXF-401) were used to evaluate the effectiveness of these agents in an in vitro cytotoxicity assay. UIISO-BCA-1 and UIISO-BCA-2 were established from pleural effusions of postmenopausal breast cancer patients (4). MAXF-401 was established from a xenograft originating from a lung metastasis (5). UIISO-BCA-1 and MAXF-401 overexpress HER-2/neu, whereas UIISO-BCA-2 expresses only basal levels. Test agents that preferentially inhibited the growth of UIISO-BCA-1 and MAXF-401, as compared to UIISO-BCA-2, were subsequently evaluated for their ability to inhibit the tyrosine kinase activity of HER-2/neu. This strategy led to the

identification of dehydrocostus lactone and episteganangin, among others, as potential antineoplastic agents that may act via inhibition of HER-2/neu.

Both compounds inhibited autophosphorylation of HER-2/neu in a dose-dependent manner. To determine the specificity of these agents toward other protein kinases, both were evaluated for effects on protein kinase C activity. Neither compound had any effect on the basal enzymatic activity of protein kinase C. However, dehydrocostus lactone did increase the enzymatic activity of phorbol ester-induced PKC. Episteganangin had no effect against the tyrosine kinase activity of a crude membrane fraction from HL-60 human leukemia cells; however, a high concentration of dehydrocostus lactone did inhibit HL-60 tyrosine kinase activity. A dose-dependent decrease in the tyrosine phosphorylation of HER-2/neu in UISO-BCA-1 cells was observed after exposure to either dehydrocostus lactone or episteganangin.

In order to obtain adequate compound for in vivo studies, several milligrams of dehydrocostus lactone were isolated from root material of *Saussurea lappa*. Both dehydrocostus lactone and episteganangin were evaluated for activity against solid tumors (UISO-BCA-1 cells) implanted into nude mice. No statistically significant inhibitory effect was observed in mice treated with episteganangin. At the dosages administered, dehydrocostus lactone did not produce a statistically significant reduction in tumor growth; however, there was some indication of tumor reduction and no gross toxicity was observed, suggesting that the compound may be effective at higher doses. Immunohistochemical studies on paraffin-embedded tumor sections confirmed the antiproliferative activity of dehydrocostus lactone.

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MEETINGS ATTENDED

Thirtieth Annual Medicinal Chemistry Meeting-in-Miniature, Iowa City, IA, May 17-19, 1992.

Tenth Marquette Life Sciences Symposium, Milwaukee, WI, March 12-13, 1993.

Thirty-fourth Annual Medicinal Chemistry Meeting-in-Miniature, Minneapolis, MN, April 23-25, 1993.

Functional Foods for Health (UIC/UIUC Joint Program), Second Annual Retreat, Monticello, IL, May 10-12, 1993. Abstract presented.

Sixth Annual Chicago Signal Transduction Symposium, Chicago, IL, May 18, 1993.

34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18-22, 1993. Abstracts presented (2).

Third Drug Discovery and Development Symposium, San Diego, CA, July 22-24, 1993.

206th Annual Meeting of the American Chemical Society, Chicago, IL, Aug. 22-28, 1993. Abstract presented.

Thirty-second Annual Medicinal Chemistry Meeting-in-Miniature, Chicago, IL, April 20-22, 1994. Meeting co-chair.

Functional Foods for Health (UIC/UIUC Joint Program), Third Annual Retreat, Monticello, IL, May 8-10, 1994.

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37th Annual Meeting of the American Society of Pharmacognosy, Santa Cruz, CA, July 27-31, 1996. Abstracts presented (2).
88th Annual Meeting of the American Association for Cancer Research, San Diego, CA, April 12-16, 1997. Abstracts presented (2)

Research Projects of Margaret R. Waltz (Preceptor: Dr. E.P. Cohen)

Title: Title: Immunity to Polymorphic Epithelial Mucin (PEM) Inhibits the Progression of Mammary Carcinoma.

Description

Introduction

This investigation was prompted by the finding that PEM, a product of the MUC1 gene, is aberrantly expressed by human breast cancer cells. PEM is a heavily glycosylated macromolecule present on the surface of both normal and malignant cells. The glycosylation pattern is altered in malignant cells. As a consequence, the altered PEM becomes immunogenic. PEM expressed by breast cancer cells has been identified as a weakly immunogenic, tumor-associated antigen (TAA). The challenge is to induce immunity to PEM in patients with breast cancer.

In a mouse model system, we tested methods found previously to augment the immunogenic properties of murine TAAs to induce immunity to human breast cancer-associated mucin. The successful application of these techniques will form the basis of analogous approaches in breast cancer patients. The methods represent an immunotherapeutic approach that can be applied to patient care. The long-term objective of the study is to develop a vaccine useful in the treatment of patients with breast cancer.

We hypothesized that presentation of human breast cancer-associated mucin in the microenvironment of immune-augmenting cytokines will lead to induction of cellular immunity to breast cancer cells in patients with the disease.

The investigation was performed in an animal model of breast cancer that mimics as closely as possible breast cancer in humans. The studies are made possible by the development in Professor Joyce Taylor-Papadimitriou's laboratory (ICRF Laboratories, London, England) of human MUC1 transgenic Sac II mice (H-2^k) that express human PEM in a tissue-specific manner. The transgenic mice are, therefore, naturally tolerant of human PEM. E3 cells, a derivative of 410.4 mouse breast carcinoma cells (BALB/c origin, H-2^d) were genetically modified to express human PEM. Thus, E3 cells express human PEM and MUC1 transgenic Sac II mice express PEM as "self."

Background

The MUC1 gene codes for a membrane mucin (PEM) expressed at the apical surface of most glandular epithelial cells. Expression is dramatically up-regulated in breast and ovarian cancer cells. The extracellular domain of mucin consists of tandem repeats of 20 amino acids with multiple O-glycans covalently bonded to the amino acid core. In breast cancer, the composition of the carbohydrate side chains is altered, resulting in the exposure of normally cryptic peptide epitopes. Aberrantly expressed breast cancer-associated mucin can become highly immunogenic, leading to the induction of T cell-mediated immunity to the malignant cells. Thus, methods to increase the

immunogenic properties of PEM could have important therapeutic implications in the treatment of breast cancer patients.

Increasing the immunogenicity of breast cancer-associated mucin: The protein core of PEM (the product of the MUC1 gene) expressed by breast cancer cells is aberrantly glycosylated, exposing a part of the protein core. The exposed core is potentially immunogenic. However, like other neoplasms, there is an immune selection in the host for malignant cells that express weakly immunogenic TAAs. (It is likely that cells that express highly immunogenic TAAs are recognized by the host's immune system and rejected.) Breast cancer cells that express weakly immunogenic PEM fail to be recognized, evade destruction by the immune system, and proliferate without apparent inhibition.

Many techniques have been used to increase the immunogenicity of malignant cells. More recent attempts include the introduction of genes specifying immune-augmenting cytokines. Tumor cells modified to form cytokines are being tested in Phase I clinical trials for patients with different types of cancer. As a consequence, there is an increase in the activation and proliferation of cytotoxic T lymphocytes (CTLs) and Natural Killer/Lymphokine Activated Killer (NK/LAK) cells directed toward the neoplastic cells.

We are evaluating this approach in mice with breast cancer. Background studies were performed in 410.4 cells, a mouse neoplasm of mammary epithelial cells arising in BALB/c mice (H-2^d). E3 cells are a derivative of 410.4 cells that were transfected with the human MUC1 gene. They express human PEM. Both 410.4 cells and E3 cells have been transfected with genes specifying immune-augmenting cytokines. The survival of mice with breast cancer is enhanced if the animals are treated by immunization with cells that secrete cytokines, or express B7.1.

Development of a colony of MUC1 transgenic mice: Professor Taylor-Papadimitriou kindly provided us with a breeding pair of MUC1 transgenic Sac II mice (H-2^K). After a six-week period of isolation in our animal colony, the mice, found to be disease-free, were released by our veterinarians to the general animal colony. The Sac II mice were then bred with BALB/c mice (H-2^d) to generate H-2^{K/d} F1 hybrids for use in the experiments. Presently, we have 72 Sac II transgenic X BALB/c F1 mice on-hand. Breeding is continuing until we have sufficient numbers of mice to begin our immunotherapy experiments. The initial studies are designed to determine the immunotherapeutic properties of E3 cells, genetically modified for cytokine-secretion in syngeneic transgenic mice with breast cancer.

Confirmation that E3 cells are syngeneic in Sac II transgenic X BALB/c F1 mice: Our initial experiments were designed to determine if E3 cells were syngeneic in Sac II transgenic X BALB/c F1 mice. The test was performed by measuring the transplantability of the genetically modified cells. (Incompatible cells are rejected.) Our results indicated that E3 cells, as well as 410.4 cells, formed tumors and grew progressively in Sac II transgenic X BALB/c F1 mice. The data indicate that both cell types are considered as "self" by the F1 mice.

Experiments in BALB/c mice with 410.4 adenocarcinomas: During the time we were breeding larger numbers of Sac II transgenic X BALB/c F1 mice, we undertook a series of necessary background experiments designed to determine the immunogenic properties of 410.4 cells modified for cytokine secretion in syngeneic BALB/c mice. The following results were obtained:

a. Cytokine secretion by transfected breast carcinoma cells.

E3 and 410.4 cells were successfully transfected with vectors carrying genes specifying immune-augmenting cytokines. The results are as follows:

Transfection of Murine Breast Cancer Cells with Vectors Specifying Cytokine Genes:

Vector	<u>Cell Type</u>	
	410.4 cells	E3 cells
pZipNeoIFN-g	transfected	(in selection)
pZipNeoIL-4	transfected	transfected
pZipNeoIL-2	transfected	transfected
MFG-mIL-12	transfected	transfected*.

*IL-12 Secretion by 410.4 Breast Cancer Cells Transfected with MFG-mIL-12

<u>Cell Type</u>	<u>IL-12/10⁶ cells/48 hrs</u>
Cells transfected with MFG-mIL-12 (410.4-IL-12)	600 pg + 15
Cells transfected with SV-(X) (410.4-SV-X)	0.0 pg
Non transfected 410.4 cells	0.0 pg

An ELISA (Endogen) specific for the p70 heterodimer of IL-12 was used in the analysis.

b. Comparison of the growth of 410.4 and E3 cells in MUC1 transgenic Sac II X BALB/c mice F1 mice.

As stated previously, both 410.4 and E3 cells of BALB/c origin are syngeneic in both BALB/c and Sac II X BALB/c F1 mice. Before investigating the immunotherapeutic properties of cytokine-secreting E3 cells, a preliminary experiment was carried out to determine the growth and time to tumor-development of 410.4 cells and E3 cells in F1 mice. The following results were obtained:

	<u>Cell Type</u>	
	<u>410.4</u>	<u>E3</u>
No. cells injected	Time to first appearance of tumor (days)*	
10 ⁶	8+2	8+2.5
10 ⁴	22+3	24+2

Varying numbers of 410.4 or E3 cells from in vitro culture were injected into breast tissue of Sac II transgenic X BALB/c F1 female mice. The time until the first appearance of a palpable neoplasm was determined. The rate of subsequent tumor growth (two-dimensional measurements) is now under evaluation.

c. The growth of 410.4-IL-12 cells in BALB/c mice.

410.4 cells are an adenocarcinoma cell line syngeneic in BALB/c mice. The cells were modified for IL-12 secretion by transduction with a vector (MFG-IL-12) specifying IL-12 (410.4-IL-12 cells). After confirmation of IL-12 secretion, 10⁶ 410.4-IL-12 cells and (for comparison) an equivalent number of unmodified, non cytokine-secreting 410.4 cells were injected into breast

tissue of naive BALB/c female mice. Tumor growth occurred in both instances. However, the rate of tumor development in mice injected with 410.4-IL-12 cells was less than that of mice injected with the unmodified 410.4 cells.

Tumor growth in BALB/c mice injected with a mixture of 410.4-IL-12 and 410.4 cells. The potential immunotherapeutic properties of 410.4-IL-12 cells were determined in BALB/c mice, syngeneic with the neoplasm. In the experiment, BALB/c mice were injected s.c. with a mixture of 1×10^6 410.4 cells and an equivalent number of 410.4-IL-12 cells. As controls, the mice received an s.c. injection of 10^6 410.4 cells or 10^6 410.4-IL-12 cells alone. The rate of tumor growth in mice injected with the mixture of 410.4 cells and 410.4-IL-12 cells was not significantly different ($P < .05$) than that of mice injected with 410.4 cells alone. Mice injected only with 410.4-IL-12 cells also developed tumors, but the rate of tumor growth in these mice was significantly ($P < .01$) less than that of mice injected with either 410.4 cells or the mixture of 410.4 cells and 410.4-IL-12 cells.

Thus, 410.4-IL-12 cells retained their tumorigenic properties in syngeneic BALB/c mice. Mice injected with a mixture of 410.4 cells and 410.4-IL-12 cells, like mice injected with 410.4-IL-12 cells alone, developed progressively growing neoplasms.

We conclude that modification of 410.4 cells for IL-12-secretion was not sufficient to eliminate the cells' tumorigenic properties.

Survival of BALB/c mice injected with 410.4-IL-12 cells. 410.4 cells modified for IL-12 secretion (410.4-IL-12 cells) exhibited tumorigenic properties in BALB/c mice. To determine if the cells were rejected after an initial period of growth, or if progressive tumor development would lead eventually to the animals' death, immunocompetent BALB/c mice were injected s.c. with 10^6 410.4-IL-12 cells or with an equivalent number of unmodified 410.4 cells. The period of survival of BALB/c mice injected with 410.4 cells was significantly ($P < .01$) less than that of mice injected with 410.4-IL-12 cells; however, in both instances, progressive tumor growth occurred until 100% of the animals succumbed to the disease.

Survival of BALB/c mice immunized with (irradiated) 410.4-IL-12 cells following a challenging injection of 410.4 adenocarcinoma cells. The potential immunogenic properties of 410.4-IL-12 cells against the growth of 410.4 adenocarcinoma cells were determined in BALB/c mice. Since our prior results indicated that the injection of 410.4-IL-12 cells resulted in the formation of slowly growing, progressive neoplasms in the mice, the cells were subjected to 5,000 rads X-irradiation before injection to prevent cell-growth. We wished to determine if the antigenic properties of the irradiated cells were preserved.

In the experiment, naive mice were immunized by injection s.c. of 10^6 410.4-IL-12 irradiated cells. (The cells received 5,000 rads X-irradiation [from a ^{60}Co source] immediately before injection.) The mice received two subsequent injections at weekly intervals of an equivalent number of irradiated 410.4-IL-12 cells. One week after the last injection, the mice were challenged by an injection s.c. of 10^6 viable 410.4 cells. As controls, other naive BALB/c mice were immunized according to the same injection schedule with an equivalent number of irradiated (5,000 rads) 410.4 cells or irradiated (5,000 rads) 410.4 cells transfected with pSVNeo(X) (specifies neomycin-resistant, but not IL-12) before the challenging injection of 410.4 cells. The results indicate that none of the mice immunized with 410.4-IL-12 cells developed tumors of 410.4 cells. The mice survived indefinitely. In contrast, mice immunized with (non cytokine-secreting) irradiated 410.4 cells or with 410.4 cells transfected with pZipNeoSV(X) before the challenging

injection of viable 410.4 cells developed progressively growing neoplasms that led eventually to the animals' death.

Thus, the immunogenic properties of 410.4-1 L.12 cells were preserved following irradiation. The cells exhibited immunotherapeutic properties against the growth of viable 410.4 breast adenocarcinoma cells in syngeneic mice.

Long-term immunity to 410.4 cells developed in mice immunized with irradiated 410.4-IL-12 cells. The results of the experiment presented in Section 4 above indicated that immunizations with irradiated 410.4-IL-12 cells protected BALB/c mice against the development of tumors of 410.4 adenocarcinoma. One hundred percent of immunized mice challenged by an injection of viable 410.4 cells appeared to have rejected the cancer cells and survived indefinitely (more than 90 days).

The possible long-term immunotherapeutic properties of 410.4-IL-12 cells were determined by rechallenging the surviving mice with a second injection of viable 410.4 adenocarcinoma cells, 90 days after the first immunization. The results indicate that all of the mice that began immunization 90 days previously with the irradiated 410.4-IL-12 cells, and survived the challenge of viable 410.4 cells, survived after a second injection of 410.4 cells. Under similar circumstances, one hundred percent of naive BALB/c mice injected with an equivalent number of 410.4 cells alone developed progressively growing neoplasms that led eventually to the death of the animals.

Growth of 410.4-IL-12 cells in BALB/c nude mice. As described, 410.4-IL-12 cells retained their tumorigenic properties in syngeneic, immunocompetent BALB/c mice; however, the rate of tumor development was less than that in mice injected with unmodified 410.4 cells. Irradiated 410.4-IL-12 cells were immunotherapeutic toward the breast cancer cells as indicated by the finding that immunizations with the irradiated cells led to long-term immunity toward unmodified 410.4 cells in BALB/c mice.

To determine the relative growth rates of 410.4-IL-12 and 410.4 cells, 410.4-IL-12 cells and, for comparison, 410.4 cells transfected with pZipNeoSV(X) were injected s.c. into BALB/c nude mice, and the rate of tumor growth of the two cell-types was compared. Both 410.4-IL-12 and 410.4 cells formed progressively growing tumors in the immune-deficient mice. Unlike tumor growth in BALB/c mice, the rate of tumor growth of 410.4 and 410.4-IL-12 cells was the same in the immune-deficient animals.

CONCLUSIONS

We interpret these findings as follows:

1. Like other neoplasms, 410.4 breast adenocarcinoma cells expressed weakly immunogenic TAAs.
2. Modification of 410.4 cells for IL-12 secretion modified, but did not eliminate, the cells' tumorigenic properties.
3. The antigenic properties of 410.4-IL-12 cells were retained if the cells were subjected to 5,000 rads X-irradiation.
4. Immunization of BALB/c mice with irradiated 410.4-IL-12 cells conferred long-term immunity to the breast adenocarcinoma.
5. IL-12-secretion followed transfection of E3 cells with MFG-mIL-12.

Other Related Work**Background.**

The human MUC1 gene is mapped to chromosome 1q21. It specifies polymorphic epithelial mucin (PEM). Expression of the MUC1 gene is up-regulated in breast carcinoma cells. The MUC1 gene is a site of frequent dysregulation in breast cancer cells, resulting in the formation of altered gene products that can potentially act as tumor associated antigens. Modification of the "normal" glycosylation pattern frequently occurs, resulting in "exposure" of the tandem amino acid repeat of the molecule. The immunity is directed toward the protein core, the polymorphic, variable number of tandem repeat region (VNTR).

The finding that there is polymorphism of the CA repeat (microsatellite) sequence within intron 6 of the MUC1 gene enabled us to examine both the microsatellite and the VNTR region of the MUC1 gene in paired DNA samples from the neoplastic and nonneoplastic cells of the same breast cancer patient. The objective was to determine if the MUC1 gene was a site of unusual alteration in human breast cancer cells. To accomplish this goal, we investigated paired DNA samples from the normal and malignant cells of 118 breast cancer patients for alterations of the MUC1 gene and the microsatellite region within the gene. The results have important implications for our understanding of the genetic mechanisms leading to the origin of TAAs specified by the MUC1 gene in breast cancer cells.

The data and details of the methods we used are presented in the enclosed manuscript (galley proofs) supported in part by the award. The results of the study indicated that the frequency of alteration at the microsatellite within the MUC1 locus was significantly ($P < .001$) higher than the frequency of alteration at two other genetic loci (D1S104 and APO-A2) not known to be involved in breast cancer. An analysis of the VNTR region within the MUC1 gene revealed an amplification of one allele in 34 of 54 (63%) informative (heterozygotes) cases. However, there was no significant association between alterations at the microsatellite within the MUC1 locus and the VNTR region within the same locus, indicating that independent genetic mechanisms were responsible for the changes. Thus, the MUC1 gene at 1q21 is a genetically unstable region. The instability is consistent with the high frequency of expression of the gene-products in breast cancer cells.

Publications during the period of this fellowship

Waltz, M.R., Pandelidis, S.M., Pratt, W., Barnes, D., Swallow, D.M., Gendler, S.J., Cohen, E.P. A microsatellite within the MUC-1 locus at 1q21 is altered in the neoplastic cells of breast cancer patients. (In Press, manuscript enclosed).

Sun, T., Kim, T.S., Waltz, M.R., Cohen, E.P. Interleukin-2 secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Cancer Gene Therapy 2(3):183-190, 1995.

Abstracts Presented

Waltz M, Pandelidis S, Pratt W, Barnes D, Hand R, Das Gupta TK, Gendler SI, Cohen EP. Alterations in Mucin by PCR and Southern Blotting. 3rd International Workshop on Carcinoma-Associated Mucins, Cambridge, England

Cohen EP, Kim TS, Sun T, Waltz MR. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from different mouse neoplasms induce tumor-specific immune responses that prolong the survival of tumor-bearing mice. Cold Spring Harbor Symposia, Conference on Gene Therapy. New York, 1994.

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specific immune responses that prolong the lives of tumor-bearing mice. Gene Therapy of Cancer, San Diego, 1994.

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Prior publication

Waltz, M.R., Pullman, T.N., Takeda, K., Sobieszczyk, P., Refetoff, S. (1990) Molecular basis for the properties of the thyroxine-binding-globulin-slow variant in American Blacks. J. Endocrinological Investigation 13:343-349. Meetings

1996 Annual Meeting of the American Association for Cancer Research April 20-24, 1996. Washington, D.C. Poster: "Non-random alterations of microsatellite DNAs in the neoplastic cells of breast cancer patients"

Histopathobiology of Neoplasia July 9-16, 1995. Keystone, CO Poster: "Non-random alterations of microsatellites in breast cancer"

Nature Genetics DNA Integrity and Instability April 6-7, 1995. Chicago, IL

3rd International Workshop on the Carcinoma-Associated Mucins August 7-11, 1994. Cambridge, England Poster: "Alterations in mucin by PCR and Southern blotting"

Additional Training

Also, during the first year of the grant, Ms. Waltz was selected to be a participant in a week-long course sponsored by the American Association for Cancer Research, titled "Histopathology of Neoplasia."

Research Projects of Lavanya Lall (Preceptor: Dr. R.L. Davidson)

Title: Sequence-specific mutagenesis in human oncogenes

Description

In the previous report, we showed that thymidine (dT) preferentially misincorporated opposite the 3'G as opposed to the 5'G of the GG doublet in codon 12 of the human ras oncogene and in certain codons of the human p53 gene that have been implicated in human breast cancer. This pattern corresponds to the most frequent mode of activation of the ras genes in human tumors, through GC -> AT transitions at the 3'G of the GG doublet in codon 12 of the human ras oncogenes, and also to a very frequent mode of activation of the p53 gene in human breast tumors. Our data suggested that dT-induced mutations might be a source of genetic alterations in the human ras and p53 genes leading to various human cancers.

As a continuation of this work, we have studied some more codons in the p53 gene that contain GG doublets and that have been implicated in human breast cancers and other human tumors, to determine the susceptibility to mispairing of the guanine residues within these doublets. Analyses of these data are underway.

We have also conducted new dT misincorporation experiments on single G residues with all possible different upstream and downstream base combinations, in order to determine the effects of various neighboring bases on the mutability of these guanines. From our results, it was determined that the guanine residue most susceptible to dT mutagenesis was the one that had

another G upstream and a T downstream (i.e., the 3'G of a GG doublet that was flanked by a downstream T). The least mutable guanine was the one that had an upstream A and a downstream C. Thus, our studies have helped define short-range sequences that may be considered "hot spots" of mutations (i.e., the sequence GGT) or "cold spots" of mutations (i.e., the sequence AGC).

In our earlier report, we had demonstrated that different bases upstream of the GG doublet had an effect on the extent of dT misincorporation opposite the 5'G of the doublet in otherwise identical sequences. It had been demonstrated that a C upstream of the GG doublet caused higher dT misincorporation opposite the 5'G of the doublet compared to an upstream T. dT misincorporation opposite the 5'G of the GG doublet was least when A was the base immediately upstream of the doublet. We had suggested that one determinant of the mutability of a particular guanine residue, with respect to its flanking neighboring bases, could be the effect of these neighbors on the stability of the G:T mispair, once it has formed. To test this possibility, we synthesized and purified DNA molecules that have G:T mispairs at either the 3' or 5' positions of the GG doublet as well as different bases upstream of the doublet. These sequences have subsequently been subjected to a range of high temperatures in order to determine the temperature at which each one undergoes "melting." This in turn would be a measure of their stability as determined by the presence of the G:T mispair as well as the identity of the base upstream of the GG doublet. These experiments are in progress.

PUBLICATIONS

Sequence directed mutagenesis in human oncogenes. Lall, L. and Davidson, R.L. Submitted.

MEETINGS ATTENDED

Presented a poster titled "Sequence directed mutagenesis in human oncogenes" at the 1997 Salk Institute/EMBL Meeting on "Oncogenes and Growth Control," La Jolla, CA.

Presented a poster titled "Sequence specific mutagenesis in human oncogenes." 12th Annual University of Illinois Molecular Biological Retreat. Lake Geneva, Wisconsin, September 9th, 1995.

7. Conclusions

We are pleased with the projects developed by the trainees and the rewarding results of each project. The results of this research were presented at national scientific meetings and submitted to peer-reviewed journals for publication.

We chose trainees from widely varied fields (Medicinal Chemistry & Pharmacognosy, Microbiology and Immunology, and Genetics), to attack the problem of breast cancer from many directions. All three trainees were women, affirming our commitment to recruiting more minorities and women into the translational research arena.

We have trained three excellent researchers who have bright futures making inroads in the area of breast cancer research. Dr. Shamon this year received her Ph.D. in Pharmacology and is now working as a Research Assistant for Dr. Ruth Lupu at the Geraldine Brush Cancer Research Institute in San Francisco. Ms. Waltz was supported as a predoctoral student through April 30, 1997; due to sudden relocation for personal reasons, she instead completed her Master's and is pursuing work in tumor immunology. Ms. Lall continues her doctoral studies in the Department of Genetics; she expects to complete her thesis by December 1998 and aims to pursue a career in cancer biology.

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Lall L. "Sequence directed mutagenesis in human oncogenes." 1997 Salk Institute/EMBL Meeting on "Oncogenes and Growth Control," La Jolla, CA.

Meetings attended

Lisa Shamon

Thirtieth Annual Medicinal Chemistry Meeting-in-Miniature, Iowa City, IA, May 17-19, 1992.

Tenth Marquette Life Sciences Symposium, Milwaukee, WI, March 12-13, 1993.

Thirty-fourth Annual Medicinal Chemistry Meeting-in-Miniature, Minneapolis, MN, April 23-25, 1993.

Functional Foods for Health (UIC/UIUC Joint Program), Second Annual Retreat, Monticello, IL, May 10-12, 1993. Abstract presented.

Sixth Annual Chicago Signal Transduction Symposium, Chicago, IL, May 18, 1993.

34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18-22, 1993. Abstracts presented (2).

Third Drug Discovery and Development Symposium, San Diego, CA, July 22-24, 1993.

206th Annual Meeting of the American Chemical Society, Chicago, IL, Aug. 22-28, 1993. Abstract presented.

Thirty-second Annual Medicinal Chemistry Meeting-in-Miniature, Chicago, IL, April 20-22, 1994. Meeting co-chair.

Functional Foods for Health (UIC/UIUC Joint Program), Third Annual Retreat, Monticello, IL, May 8-10, 1994.

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Margaret Waltz

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Histopathobiology of Neoplasia July 9-16, 1995. Keystone, CO Poster: "Non-random alterations of microsatellites in breast cancer"

Nature Genetics DNA Integrity and Instability April 6-7, 1995. Chicago, IL

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Lavanya Lall

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9. List of Personnel Receiving Pay From This Grant

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10. Appendix 1

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A Microsatellite within the MUC1 Locus at 1q21 is Altered in the Neoplastic Cells of Breast Cancer Patients

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Margaret R. Waltz, Steven M. Pandelidis, Wendy Pratt, Diana Barnes, Dallas M. Swallow, Sandra J. Gendler, and Edward P. Cohen

ABSTRACT: Paired DNA samples from the neoplastic and nonneoplastic cells of 118 patients with the sporadic, nonfamilial form of breast cancer were analyzed for evidence of genetic alteration at a polymorphic microsatellite mapped to intron 6 within the MUC1 gene at 1q21. Two other microsatellite loci, D1S104 and APO-A2, which also map to 1q21, were analyzed as well. The frequency of alteration at the microsatellite within the MUC1 locus was significantly higher than D1S104 or APO-A2 ($P < 0.001$). Analysis by Southern blotting of the VNTR region of the MUC1 gene revealed an amplification of one allele in 34 of 54 informative cases (63%). There was no significant association between these alterations and alterations of the microsatellite within the same locus, suggesting independent mechanisms were responsible for the genetic changes. Microsatellite loci D17S579 at 17q21, the site of the BRCA-1 gene, and D18S34 at 18q21-qter, the deleted in colorectal cancer locus, were also analyzed by PCR. Alterations at D17S579 and D18S34 were detected in 18.8% and 6.2% of patients, respectively ($P < 0.001$, and $P < 0.1$ relative to the frequency of alteration at D1S104 or APO-A2). A previously described polymorphism of hMSH2 was altered in 16.4% of cases. © Elsevier Science Inc., 1997

INTRODUCTION

An accumulation of genetic changes within an individual somatic cell is responsible for conversion to the malignant phenotype [1-4]. As the malignant cell population increases, genetic instability often results in the appearance of neoplastic cells with varying abnormal properties, such as the capacity for metastasis, and resistance to drugs commonly used in cancer treatment [5-8].

Various genetic loci have been identified that are frequently altered in neoplastic cells. Certain changes are associated with the malignant phenotype. The BRCA-1 gene at 17q21, and the VNTR region of the MUC1 gene at 1q21 are notable examples [9-11]. A high frequency of alterations in microsatellite sequences [12-17] in genomic DNA from the tumor can be a first indication of the presence of significant genetic change in neoplastic cells, and may lend insight into the genetic mechanisms involved in mediating the alterations [15].

Pratt et al. [18], recently described the polymorphism of a microsatellite (CA-repeat) sequence within intron 6 of the MUC1 gene. The gene includes a polymorphic VNTR region within exon 2 that is frequently altered in breast cancer cells [11]. Figure 1 illustrates the location of these features of the MUC1 gene. Whether the microsatellite is altered in breast cancer cells and whether such alterations are associated with alterations of the VNTR region has not been determined.

To investigate these questions, we analyzed the microsatellite and VNTR region of the MUC1 gene in paired neoplastic and nonneoplastic cells of 118 patients with the sporadic form of breast cancer. In addition, four other loci were analyzed for microsatellite instability. Two of these have been associated with tumor suppressor genes. D17S579 at 17q21 [9] and D18S34 at 18q21-qter [19], whereas the other two, APO-A2 and D1S104, are located near the MUC1 gene, at 1q21.

MATERIALS AND METHODS

Human Tissue Samples

Fresh solid tumor tissues, taken during the usual course of the patients' treatment, were dissected free of fat and stored in liquid nitrogen. Peripheral blood buffy coat cells obtained at the time of surgery were used as the source of

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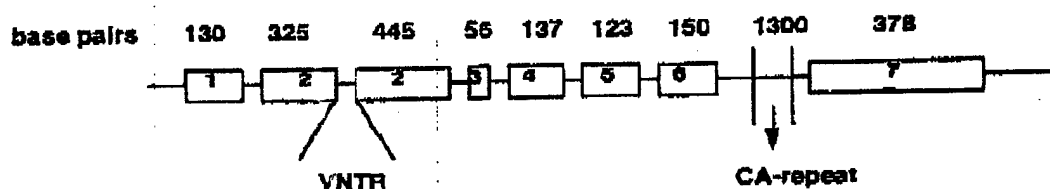


Figure 1 Diagram of the MUC1 gene. The variable number tandem repeat region (VNTR) is located within exon 2 of the gene. The CA repeat is located within exon 8 of the gene.

DNA from nonneoplastic cells from the same individuals. Confirmation of the malignant nature of the tumor was determined in stained paraffin-embedded sections, prepared according to conventional techniques.

Preparation of High-Molecular-weight DNAs From Paired Neoplastic and Nonneoplastic Cells of the Same Individuals With Breast Cancer

Frozen tumor tissues (approximately 0.3 gms) were disaggregated in a Braun (Melsungen, Germany) Mikro-disembrator II, and the DNA was isolated from the cells in an Applied Biosystems (Foster City, CA) 340A DNA extractor. Nonneoplastic cells from the patient's peripheral blood were lysed with water before addition to the extractor. After dialysis, the DNA concentrations were measured spectrophotometrically. Before the various analyses were performed, aliquots of undigested DNA from the paired samples were subjected to electrophoresis through 0.7% agarose gels. Degraded specimens were not included.

Analysis of Microsatellite DNAs From Paired Neoplastic and Nonneoplastic Specimens of the Same Patients

Analyses of each of five microsatellite loci were performed by PCR, using oligonucleotide primers that flanked the region of interest. The loci investigated and the primer sequences are presented in Table 1. Fifty-microliter reaction mixtures consisted of 300 ng of DNA 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1% Triton X-100), 2 mM MgCl₂, 1.25 mM of each dNTP, 50 pMol of each primer, 3 U of Taq polymerase (Promega, Madison, WI). The samples were overlaid with oil, placed in a thermocycler (Perkin-Elmer, Norwalk, CT) and subjected to 27 cycles of 94°C, 45 seconds; 55°C, 45 seconds; 72°C, 45 seconds. That the PCR

products were of the correct size was confirmed by agarose gel electrophoresis. The PCR products were separated in 8% polyacrylamide denaturing gels and then exposed to XAR-5 film (Kodak, Rochester, NY) at -70°C for 1 to 3 days.

The autoradiographs were compared for differences in the microsatellites from the neoplastic and nonneoplastic cells of the same patients. Changes in the size of a band, deletion or addition of a band in the DNA from the tumor, compared to DNA from the nonneoplastic cells, were considered as evidence of alterations.

Analysis of the VNTR Region of the MUC1 Gene at 1q21 in the Neoplastic and Nonneoplastic Cells of Breast Cancer Patients

Southern blotting was used to detect possible alterations in the polymorphic VNTR region of the MUC1 gene in the neoplastic cells of the breast cancer patients. Approximately 10 µg of DNA from the neoplastic or nonneoplastic cells of the same patients was digested to completion with HinfI (Gibco BRL, Gaithersburg, MD), and fractionated in 0.7% agarose gels. HindIII digested lambda DNA (Gibco BRL, Gaithersburg, MD) was used as a molecular-weight marker. After electrophoresis, the agarose gels were denatured in alkali and transferred to nylon membranes (Bio-dyne, Pall, Glen Cove, NY). After transfer, the membranes were incubated for 1 hour at 80°C under vacuum, and then hybridized with a probe homologous to the VNTR region at exon 2 of the MUC1 gene [20]. The probe was labeled to highly specific activity with [α -³²P]-dCTP by the random priming method [21]. Hybridization was performed at 42°C in the presence of nonspecific DNA (herring testis, Promega, Madison, WI) in a buffer containing 5X SSC (1X SSC in 150 mM NaCl, 15 mM trisodium citrate, (pH 7.0), and 50% formamide). After incubation, the filters were

Table 1 Sequence of primers used for analysis of microsatellites

Microsatellite	Primer Sequence	Reference
D17S579	5'-AGT CCT GTA GAC AAA ACC TG 5'-CAG TTT CAT ACC AAG TTC CTA	[15]
D18S34	5'-CAG AAA ATT CTC TCT GGC TA 5'-CTC ATG TTC CTG GCA AGA AT	[17]
APO-A2	5'-GGT CTG GAA GTA CTG AGA AA 5'-GAT TCA CTG CYG TGG ACC CA	[10]
D18104	5'-ATC CTG CCC TTA TGG AGT GC 5'-CCC ACT CCT CTG TCA TTG TA	[23]
Ms within MUC1	5'-AGG AGA GAG TTT AGT TTT CTT GCT CC 5'-TTC TTG GCT CTA ATC AGC CC	[26]

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Table 2 Summary of microsatellite alterations

	MUC1	D17S579	D18S34	D1S104	APOA2
No. of cases	104	101	94	74	70
No. of information cases	100	90	81	65	70
	(96.1%)	(89.1%)	(87.2%)	(87.8%)	(100%)
Total no. of changes	24	17	5	3	2
	(23.8%)	(18.8%)	(6.2%)	(4.7%)	(2.8%)
LOH	5	9	3	0	0
Allelic imbalance	7	4	1	0	0
Size change	4	4	1	2	1
Additional bands	8	0	0	1	1

washed at 55°C and exposed to XAR-5 film (Kodak, Rochester, NY) at -70°C for 1 to 3 days.

Analysis by SSCP of the hMSH2 Locus in the Paired Neoplastic and NonNeoplastic Specimens of the Breast Cancer Patients

Analysis of the conserved region of hMSH2, a replication error repair gene [2], was performed according to the method described by Orita et al. [22], using oligonucleotide primers specific for codons 668-736 of the hMSH2 locus. The primers were:

PF: 5'-CGC GAT TAA TCA TCA GTG-3'
PR: 5'-GGA CAG AGA CAT ACA TTT GTA T-3'

The reaction mixture consisted of 200 ng genomic DNA, 5 ml of 10X reaction buffer (Promega, Madison, WI), 1.5 mM MgCl₂, 50 pMol of each primer, 25 nMol of each nucleotide, 10 mCi [³²P]-dCTP, and 2.5 Units Taq polymerase (Promega, Madison, WI) in a total volume of 50 µl. The reaction mixtures were cycled 27 times, at 94°C, 45 seconds; 57°C, 45 seconds; and 72°C, 45 seconds for each cycle. Afterward, 6 µl of the mixture was heated to 95°C for 3 min before it was analyzed in a neutral 7% polyacrylamide gel containing 10% glycerol. The gels were subjected to electrophoresis at room temperature for 6 hours at 50 W. They were dried and exposed to XAR-5 film (Kodak, Rochester, NY) at -70°C for 1 to 3 days. Sequencing of the PCR products was performed using a double strand DNA Cycle Sequencing System (Gibco/BRL, Gaithersburg, MD), according to the directions of the supplier.

RESULTS

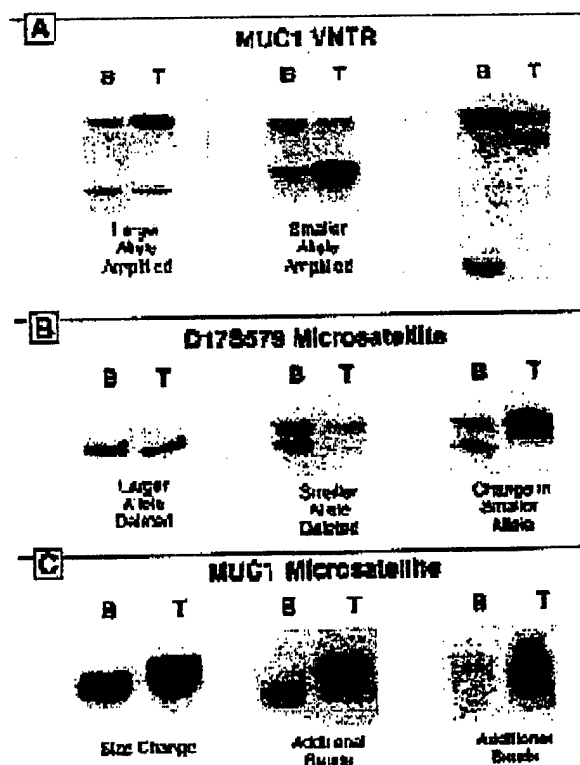
A Microsatellite at Intron 6 Within the MUC1 Gene at 1q21 is Altered in the Neoplastic Cells of Breast Cancer Patients

The microsatellite within the MUC1 gene was analyzed in the paired neoplastic and nonneoplastic specimens from 100 informative patients with the sporadic form of breast cancer. For comparison, two other microsatellite loci mapping to 1q21, and two loci at 17q21 and 18q21, which are associated with tumor suppressor genes, were also analyzed.

Table 2 indicates the changes in microsatellites observed and a breakdown of the changes seen at each locus. The numbers of Losses of Heterozygosity (LOH), size changes and additional bands present are indicated. "Al-

lelic imbalance" has been used to describe those microsatellite alterations that appear as an increase or decrease in the band intensity of one allele relative to the other allele [8]. The frequency of alteration of the microsatellite at intron 6 within the MUC1 gene at 1q21 was significantly ($P < 0.001$) higher than that of APOA2 and D1S104, the other two microsatellites at the same locus. D17S579 and D18S34 were also altered at a high frequency relative to APOA2 and D1S104, 18.8% and 6.2%, respectively. Figure 2B and C presents the types of changes observed at microsatellite loci. The majority (57%) of alterations were

Figure 2 Representative examples of alterations of the VNTR of MUC1 and microsatellite loci. "B" denotes DNA extracted from blood, and "T" denotes DNA extracted from tumor.



LOH, 23% were size changes of an allele, and 20% were indicated by the presence of additional bands.

The VNTR Region Within the MUC1 Gene at 1q21 was Altered in the Neoplastic Cells of the Breast Cancer Patients

An analysis of the polymorphic VNTR within the coding region of the MUC1 gene revealed a high frequency of alteration in the neoplastic cells of the patients. The analysis was performed by Southern blotting. Representative alterations are presented in Figure 2A. The gene was altered in 34 of 54 informative patients. There was LOH in 3 instances (5.2%), amplification of one of the two alleles in 32 cases (56%), and one translocation (1.8%). Amplification of either allele was considered as "allelic imbalance" in Table 2.

Of the three cases of LOH at the VNTR of the MUC1 gene, none showed LOH of the microsatellite in intron 6 of the gene. This was unexpected, as losses of heterozygosity tend to reflect the deletion of large segments of genetic material [26]. If such a loss had occurred at 1q21, both the VNTR and the microsatellite should show LOH. However, an alternate explanation for the LOH at the VNTR is that there has been homologous recombination of the MUC1 gene in the tandem repeat region, resulting in two alleles of the same size. If this were the case, the 1q21 locus may be present, though the VNTR of the MUC1 gene is no longer heterozygous. This would explain how the three individuals with LOH at the VNTR may also be informative for the three microsatellite markers at 1q21.

There was no significant association between an alteration of the VNTR region and an alteration of the microsatellite at intron 6 within the MUC1 gene in the neoplastic cells of the same patient ($P > 1.0$). The likelihood that patients whose neoplastic cells revealed both an alteration at the VNTR region and alteration of the microsatellite within intron 6 was not greater than random.

Alterations of the hMSH2 Gene were Not Detected in the Neoplastic Cells of the Breast Cancer Patients

The hMSH2 gene encodes a DNA repair protein that binds to base pair mismatches generated during DNA replication [2]. An analysis of the gene for hMSH2 was carried out by SSCP, using primers for codons 668-736 and flanking sequences (corresponding to bps 2072 to 2208) of the hMSH2 locus [2]. This portion of the gene is conserved across species and is thought to encode the DNA binding region of the protein. Sixteen of 97 cases examined (16.4%) revealed an alteration of the hMSH2 gene. The same alteration was present in both the neoplastic and nonneoplastic cells of the same patient. DNA sequencing was carried out to characterize the alteration in greater detail. It revealed a G to T transition at the -6 splice acceptor site. Because the alteration was found in DNA from both normal and tumor cells of the same individuals, it was likely that the variation was a germline, rather than somatic change in sequence. (These data are not presented.)

There was no significant association between a change in the hMSH2 gene and a change in any of the microsatellite loci investigated. DNA from the neoplastic cells of one

patient revealed instability of microsatellite DNA at two of the five loci. The hMSH2 gene was not altered in the neoplastic or nonneoplastic cells of this patient.

DISCUSSION

Genetic instability of malignant cells is indicated by widespread alterations in microsatellite DNAs. Because the alterations are not random, they are taken as indications of genetic changes that are involved in the malignant phenotype. Here, we present the results of a comparative analysis of five microsatellite loci and of the VNTR region of the MUC1 gene in malignant and nonmalignant cells of 118 breast cancer patients. The investigation was an extension of prior studies in which it was found that the MUC1 gene was frequently altered in the neoplastic, but not the nonneoplastic cells of patients with primary breast carcinomas [19].

Both the VNTR region of the MUC1 gene and a microsatellite within the same gene were altered in a high proportion of the patients. However, the likelihood that changes in the VNTR region were accompanied by changes in the microsatellite within the gene was not greater than random. This finding may not be unexpected, as microsatellite and VNTR loci, though both frequent sites of genetic alteration, may be subject to different types of mutation. Indeed, the data were consistent with the presence of different genetic mechanisms responsible for alteration of the VNTR region and a change in the microsatellite. Because the MUC1 gene includes tandemly repeated DNA, it is possible that alteration of the VNTR region was mediated by homologous recombination, whereas alterations in microsatellite DNA may arise from slippage during the replication of simple repetitive sequences, followed by failure of the cell to repair the damage. In support of this hypothesis is a previous finding that VNTR loci are not affected by the replication error phenotype [15]. Why the CA repeat within the MUC1 gene was altered at a higher frequency than two other microsatellites at 1q21 was not determined. Conceivably, a change in the microsatellite conferred an undefined growth advantage to the malignant cells.

The Replication Error Repair (RER⁺) phenotype has been found in HNPCC colon cancers as well as in other sporadic tumors associated with HNPCC syndrome [1/3, 23]. In our investigation, microsatellite alterations were occasionally detected, though most were losses of heterozygosity. None of the specimens revealed the ladder-like expansions seen in the microsatellite instability of colorectal cancers [1/3]. Similar changes have been noted previously in studies of patients with the sporadic form of breast cancer [23].

Fishel et al. [2] and Leach et al. [3] reported that approximately 60% of patients with colon cancer with the RER⁺ phenotype had mutations of the hMSH2 gene. We attempted to detect analogous changes at the hMSH2 locus in the specimens we examined. A T to C substitution at the -6 position of the splice acceptor site was present in 16 of 97 cases. However, because it was found in both the neoplastic as well as nonneoplastic cells of the same individuals, it was likely to have been a polymorphism. Leach et al. [3] found the same mutation in 2 of 20 tumor-free

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individuals. No significant association between this alteration at the hMSH2 locus and alterations of the microsatellite loci was detected.

We conclude that alterations in microsatellites in sporadic breast cancer are likely to result from a different mechanism from that responsible for microsatellite instability in HNPCC. In addition, we confirm the high frequency of alteration in the region of the BRCA1 locus and in the VNTR at exon 2 of the MUC1 gene in sporadic breast cancer. The high frequency of alteration of the CA repeat within the gene was an unexpected finding. Its possible involvement in progression of the tumor remains to be determined.

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A Correlative Approach for the Identification of Antimutagens that Demonstrate Chemopreventive Activity*

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Abstract. Seventy natural and synthetic compounds were tested for potential to inhibit mutation induced by 7,12-dimethylbenz(a)anthracene (DMBA) in *Salmonella typhimurium* strain TM677. Results were compared with their ability to inhibit DMBA-induced preneoplastic lesions in a mouse mammary gland organ culture system. The response mediated by fifty-five of the test compounds was either positive or negative in both test systems, indicating that the combined use of these assays should aid in the discovery of antimutagenic agents that have cancer chemopreventive potential.

Since retinoids emerged as an important class of compounds capable of exhibiting protective effects against experimental carcinogenesis (1-3), an increasing number of agents of diverse chemical structure have been studied as potential chemopreventive agents (4). Characterization of chemopreventive agents typically involves traditional carcinogenesis studies that require relatively large numbers of animals and take several months to complete. Therefore, it is important to limit such studies to the investigation of select agents which have clear potential for demonstrating chemopreventive activity.

A main objective of our research program is the discovery of novel plant constituents which are active cancer chemopreventive agents. The approach most commonly used in such drug discovery programs is bioassay-directed fractionation.

*Some of the results herein were presented at the 34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18-22, 1993.

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Key Words: Antimutation, chemoprevention, mammary gland, organ culture, *Salmonella typhimurium*.

In general, this entails the screening of plant extracts for potential to mediate an appropriate biological response, followed by successive fractionation and biological evaluation of resulting fractions until pure active isolates are obtained (5). One way of accomplishing this objective is to utilize *in vitro* evaluation systems that can provide an early indication of the potential of test agents to inhibit carcinogenesis. Mammary gland organ culture is one such *in vitro* test system. It has been demonstrated that the mammary gland organ culture mimics hormonally regulated physiological stages observed in animals (6,7).

Additionally, experimental evidence indicates that 7,12-dimethylbenz(a)anthracene (DMBA)-induced lesions are preneoplastic in nature since transplantation of cells from the *in vitro* mammary lesions form adenocarcinomas upon transplantation into syngeneic mice (8,9). Moreover, there is excellent correlation between inhibitors of preneoplastic lesions in the *in vitro* mammary organ culture test system and inhibition of carcinogen-induced mammary tumors in rodents (10,11). Thus, this *in vitro* test system provides a valid model for the evaluation of pure compounds of putative chemopreventive value. Although it is possible to use mammary organ culture for bioassay-directed fractionation schemes described above, fractionation of a single plant extract could require the sacrifice of over 4000 mice and a test period of over one year (12). Under normal circumstances, this is not practical.

Based on these considerations, we hypothesized that a more efficient approach would involve evaluating test materials in a rapid (but non-selective) antimutagenesis assay system and then evaluating only the positive substances in the mammary organ culture system. Materials testing positive in both systems could then be subjected to bioassay-directed fractionation utilizing the antimutagenesis test system. Presumably, the resulting isolates would be likely to mediate a positive response in the mammary organ culture system which, as stated above, correlates with *in vivo* tumor inhibition. In order to test this hypothesis, 70 test compounds were evaluated for their ability to inhibit DMBA-induced mutation

with *Salmonella typhimurium* strain TM677, and the results were compared to the ability of the compounds to inhibit DMBA-induced mammary lesions in organ culture. As described herein, sufficient association of activities was established to conclude that this approach should be useful for the discovery of antimutagens capable of demonstrating chemopreventive activity.

Materials and Methods

Materials. Allylmethyl disulfide, diallyl sulfide and silymarin were purchased from Aldrich Chemical Co. (Milwaukee, WI); BASF 47850, BASF 47343, 4-hydroxyphenylretinamide and etoperidone hydrochloride were obtained from the National Cancer Institute (Bethesda, MD); thiolutin was obtained from Pfizer Inc. through the National Cancer Institute; cryptoporic acid D, cryptoporic acid E, epigallocatechin gallate and morusin were supplied by Dr. H. Fujiki from the Cancer Research Institute (Tokyo, Japan); daidzein and fisetin were obtained from Indofine Co. (Somerville, NJ); brassinin was supplied by Dr. R. M. Moriarty, Department of Chemistry, University of Illinois at Chicago; deguelin and tephrosin were supplied by Dr. A. D. Kinghorn, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago; difluoromethylornithine was from Merrill Dow (Cincinnati, OH); suramin was from Bayer AG (Wuppertal, Germany); dehydrocosus lactone, linoleic acid, 9-octadecynoic acid, palmitoleic acid and ursolic acid were provided by Dr. M. E. Wall, Research Triangle Institute (Research Triangle Park, NC); Waymouth's 752/1 MB medium was purchased from GIBCO BRL (Grand Island, NY); all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Antimutagenicity assay with *Salmonella typhimurium* strain TM677. The procedure used to detect forward mutation to 8-azaguanine resistance in *Salmonella typhimurium* strain TM677 has been described in detail (13). Bacteria were cultured in a shaking water bath (1 h, 37°C, minimal essential medium). For each sample, a solution consisting of 0.77 ml of bacteria in minimal essential medium, 0.1 ml of S9 liver homogenate derived from Aroclor 1254-pretreated rats (14) and 0.11 ml of a NADPH-generating system was distributed into 15 ml conical tubes. To monitor antimutagenic activity, test substances (100 µM final concentration added in 10 µl DMSO) were added to duplicate incubation mixtures one minute prior to the addition of DMBA (80 µM final concentration added in 10 µl DMSO). Following incubation in a rotating dry-air incubator (2 h, 37°C), the reaction mixtures were quenched by the addition of 4 ml of phosphate buffered saline. The bacteria were recovered by centrifugation, resuspended in 5 ml of phosphate-buffered saline, diluted, and plated (in triplicate) on minimal agar in the presence and absence of 8-azaguanine. The plates were then incubated (48 h, 37°C) and scored with an automatic colony counter (Fisher Model 600). The results were expressed as a mutant fraction, i.e., the average number of colonies capable of growing in the presence of 8-azaguanine divided by the average number of colonies capable of growing in the absence of 8-azaguanine, after correcting for dilution factors. The percent inhibition was then calculated relative to control plates that were treated only with DMSO.

Mouse mammary gland organ culture assay. The assays were performed essentially as previously described (6,15,16). BALB/c female mice (4 weeks old, Charles River, Wilmington, MA) were pretreated for nine days with 1 µg of estradiol and 1 mg of progesterone. On the tenth day, the mice were sacrificed and the second thoracic mammary glands were dissected on silk and transferred to 60 mm culture dishes containing 5 ml of Waymouth's 752/1 MB medium supplemented with 100 units streptomycin and penicillin and 35 µg/ml glutamine. The glands were incubated for 10 days (37°C, 95% O₂ + 5% CO₂) in the presence of growth promoting hormones (5 µg insulin, 5 µg prolactin, 1 µg aldosterone and 1 µg hydrocortisone per ml of medium). Glands were

exposed to 2 µg/ml DMBA between 72 and 96 h. After the exposure, glands were rinsed and transferred to new dishes with fresh medium. The fully differentiated glands were then permitted to regress by withdrawing all hormones except insulin for 14 additional days. To evaluate the inhibition of DNA damage, chemopreventive agents (5 concentrations, ranging from 10⁻⁹ M to 10⁻⁵ M; 15 glands per treatment group) were present in the medium during days 1-10 of culture. Mammary glands were scored for incidence of lesions as previously described (16).

Results

As summarized in Table I, 70 compounds were evaluated for antimutagenic potential against DMBA, and compared with their ability to inhibit DMBA-induced preneoplastic mammary lesions in the mouse mammary gland organ culture system. Antimutagenic results are expressed as percent inhibition of the DMBA-induced mutant fraction. Based on statistical significance using Student's *t*-test (*p* < 0.025), compounds which inhibited the mutagenicity of DMBA by 25% or greater were considered active antimutagens. Mammary organ culture results are presented only as active or inactive. Percent incidence of lesions for each group was determined as a ratio of the glands positive for lesions over the total number of glands in that particular group. Percent inhibition by chemopreventive agents at each dose level was calculated. Based on statistical significance using Chi-squared analysis, compounds which inhibited mammary lesions by 60% or greater were considered active chemopreventive agents.

Thirty-eight compounds were active in both assays. Antimutagenic activity ranged from weak (25%) to total inhibition of induced mutation. Seventeen compounds demonstrated little or no activity in either assay. Ten compounds which were effective in the mammary organ culture assay demonstrated no significant antimutagenic activity. Five compounds showed antimutagenic activity but were not effective in the mammary organ culture assay.

Discussion

Obviously, the process of carcinogenesis is significantly more complicated than mutagenesis. Therefore, it is unlikely that a random screening program utilizing antimutagenicity as a sole endpoint will yield agents that are useful for the inhibition of carcinogenesis. Rather, it is important to establish close association between the antimutagenic response and other responses that are more physiologically representative of the desired endpoint, i.e., cancer chemoprevention. Toward that objective, we have tested a variety of compounds in a short-term bacterial antimutagenesis assay and compared the results with the responses obtained using mammary organ culture assay. The forward mutation assay utilizing *Salmonella typhimurium* strain TM677 is histidine-independent, making it a favorable assay system for testing plant extracts that may contain amino acids. In addition, since cell viability is factored into the antimutagenic response, bactericidal and antimutagenic effects can be distinguished. This distinction of

Table I. Inhibitory effects of potential chemopreventive agents on forward mutation of *Salmonella typhimurium* strain TM677 and correlation with effects in mammary organ culture.

Compound tested ^a	%Inhibition of DMBA-induced forward mutation (% survival)	
Agents active as antimutagens and active in mammary organ culture		
N-Acetylcysteine	64±8 ^b	(100)
Apigenin	67±5 ^b	(98)
Aspirin	73±6 ^b	(100)
BASF 47850	84±4 ^b	(96)
BASF 47343	100±0.9 ^b	(95)
Brassinin	65±6 ^b	(94)
Carbenoxolone	61±8 ^b	(100)
6-Carotene	51±9 ^b	(100)
(+)-Catechin	25±15 ^b	(93)
Chlorophyllin	99±0.1 ^b	(100)
Cineole	94±6 ^b	(1)
Curcumin	55±10 ^b	(100)
Dehydroepiandrosterone	78±5 ^b	(100)
Diallyl sulfide	73±5 ^b	(21)
Ellagic acid	46±9 ^b	(100)
Epigallocatechin gallate	77±5 ^b	(100)
Esculetin	66±6 ^b	(96)
Etoposide	27±16 ^b	(100)
Ferulic acid	45±9 ^b	(96)
6-Glycyrrhetic acid	42±10 ^b	(96)
4-Hydroxyphenylretinamide	48±11 ^b	(74)
Ibuprofen	54±11 ^b	(97)
Indomethacin	69±7 ^b	(100)
Levamisole	39±14 ^b	(100)
Limonene	89±5 ^b	(2)
Lycopene	27±10	(100)
D-Mannitol	76±4 ^b	(97)
Melatonin	29±16 ^b	(100)
Methylene blue	64±8 ^b	(69)
Miconazole	85±4 ^b	(100)
Nordihydroguaiaretic acid	83±3 ^b	(100)
Quercetin	71±6 ^b	(84)
Retinoic acid	46±12 ^b	(100)
Silymarin	63±7 ^b	(100)
Sodium meclofenamate	38±15 ^b	(100)
Sodium molybdate	49±9 ^b	(100)
Sodium selenite	100±0.2 ^b	(82)
Tetracycline	47±15 ^b	(2)
Agents inactive as antimutagens and inactive in mammary organ culture		
Allylmethyl disulfide	29±23 ^c	(73)
D-Arginine	NI ^d	(87)
Cryptoporic acid D	NI	(86)
Cryptoporic acid E	24±13 ^c	(94)
Daidzein	NI	(100)
Dehydrocostus lactone	13±11 ^c	(97)
Dibromoacetophenone	29±12 ^c	(82)
Linoleic acid	NI	(100)
p-Methoxyphenol	NI	(81)
Morusin	NI	(72)
9-Octadecynoic acid	NI	(100)
Palmitoleic acid	NI	(97)
Piroxicam	NI	(100)
Retinyl acetate	22±17 ^c	(91)
Rutin	NI	(81)
α-Tocopherol acetate	NI	(100)
Ursolic acid	NI	(100)

continued

Table I continued

Agents inactive as antimutagens and active in mammary organ culture		
Calcium glucarate	NI	(95)
Deguelin	19±13 ^c	(89)
Difluoromethylornithine	NI	(86)
DL-Methionine	NI	(90)
(-)-2-Oxothiazolidine		
4-carboxylic acid	NI	(100)
Phytic acid	NI	(81)
Sulindac	NI	(100)
Tephrosin	22±12 ^c	(88)
Thiolutin	NI	(29)
(±)-Verapamil	NI	(100)
Agents active as antimutagens and inactive in mammary organ culture		
L-Carnosine	52±12 ^b	(100)
Cysteamine	65±8 ^b	(100)
Fisetin	56±14 ^b	(99)
Menadione	67±8 ^b	(93)
Suramin	86±3 ^b	(100)

^aAll compounds were tested at a concentration of 100 μM. The values are expressed as percent inhibition of DMBA activity. Test compounds were evaluated in several different experiments: DMSO mutant fractions ranged from 6.8±0.5 to 20.1±2.2×10⁻⁵; corresponding DMBA mutant fractions ranged from 22.5±3.7 to 47.4±6.6×10⁻⁵. In each experiment, the mutant fraction observed in the DMBA-treated sample was greater than the DMSO-treated control ($P < 0.025$, Student's *t*-test).

^bSignificant at $p < 0.025$, Student's *t*-test (versus DMBA).

^cNot significant at $p < 0.025$, Student's *t*-test (versus DMBA).

^dRelative to DMBA-treated control, no inhibition was detected.

activities can be seen in many of the compounds tested, e.g., cineole, diallyl sulfide, dibromoacetophenone and tetracycline, which demonstrated strong bactericidal activity.

Out of 70 compounds tested, 55 demonstrated corresponding activity in both assay systems. These compounds comprised several chemical and functional classes of agents, including flavonoids, retinoids, thiols, anti-inflammatory agents and antioxidants. As an example, similar responses were achieved in both assay systems for all five retinoids tested. Retinoic acid, 4-hydroxyphenylretinamide and its derivatives BASF 47850 and BASF 47343 demonstrated a positive response in both systems, while retinyl acetate elicited a negative response. Out of several non-steroidal anti-inflammatory agents tested, only sulindac demonstrated noncorresponding activity. Ten compounds which were active in the mammary organ culture assay did not demonstrate antimutagenic activity. However, four of these "false-negatives," deguelin, difluoromethylornithine (17), tephrosin (18) and thiolutin (19), inhibit clonal evolution of tumors and not DNA damage. Therefore, these compounds would not necessarily be expected to mediate an antimutagenic response. On the other hand, only five of the compounds tested, L-carnosine, cysteamine, dibromoacetophenone, fisetin and menadione, were active as antimutagens without inhibiting mammary lesions.

These results indicate that the comparative approach of combining *in vitro* antimutagenesis and *in vitro* inhibition of preneoplastic mammary lesions can be utilized successfully to screen for identification of inhibitors of carcinogenesis. The association applies for a broad array of structural types and where a lack of association was observed, mechanistic insight may provide an explanation. This approach is obviously limited to the discovery and characterization of agents capable of serving as inhibitors of DNA damage; alternate *in vitro* test systems are required for the evaluation of chemopreventive agents that interfere with other stages of carcinogenesis (20). Nonetheless, based on the number of agents demonstrating antimutagenic activity (Table 1) that are known to inhibit tumorigenesis, it is reasonable to conclude that antimutagenesis may be used as an initial screen in order to select plant extracts, isolates or synthetic agents for more conclusive *in vitro* or *in vivo* carcinogenesis studies.

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Assessment of antimutagenic activity with *Salmonella typhimurium* strain TM677

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Abstract. A method is described for the detection of antimutagenic agents in a forward mutation assay with *Salmonella typhimurium* strain TM677. Bacterial cells are treated with test compounds in the presence of a known mutagen. Antimutagenic activity is indicated by a reduction in the induced

mutant fraction. This assay has been used to detect and/or confirm the antimutagenic activity of a number of known compounds. This method is currently being used in our laboratory for the bioassay-directed fractionation of potential cancer chemoprevention agents from plant extracts.

Key words: Antimutagenesis, *Salmonella typhimurium*

Abbreviations: XGPRT = xanthine-guanine phosphoribosyltransferase; BHI = brain-heart infusion; DMSO = dimethylsulfoxide; DMBA = 7,12-dimethylbenz[*a*]anthracene; PBS = phosphate buffered saline; B[*a*]P = benzo[*a*]pyrene; MEM = minimum essential medium

1. Introduction

Inhibition of mutation is one of several potentially effective mechanisms for the prevention of human cancers resulting from DNA damage due to exposure to environmental mutagens [1, 2]. Such damage is associated with cancer and genetic defects, and may contribute to conditions such as heart disease and aging [3–5]. Additionally, mutagens may be involved throughout the process of carcinogenesis, well beyond the initiation stage [6, 7]. On the other hand, a wide variety of chemical agents have been studied as potential cancer chemopreventive agents [8, 9], and many of these compounds have demonstrated antimutagenic activity [10, reviewed in 11]. Although the development of cancer obviously depends on much more than mutagenesis, a general correlation has been established between the mutagenic and carcinogenic potential of a large number of chemicals [12], and therefore antimutagenic activity is expected to be beneficial.

Since chemical agents can induce mutations by a variety of mechanisms, it is logical that a variety of substances can interfere with the mutagenic process. The most general classification of antimutagens places them into two broad categories: desmutagens and bioantimutagens. Desmutagens interact directly with mutagens [13] and deactivate them through chemical or enzymatic reactions such as inhibition of metabolic activation of a pro-mutagen, induction of detoxification enzymes, direct chemical reaction with

a mutagen, scavenging of reactive oxygen species, protection of nucleophilic sites on DNA, etc. [7]. Bioantimutagens act to induce repair of DNA damaged by mutagens or inhibit error-prone repair systems [13, 14].

The forward mutation assay with *Salmonella typhimurium* strain TM677 was originally devised by Professor William G. Thilly and co-workers at Massachusetts Institute of Technology [15]. This assay has been used successfully in our laboratory to evaluate the mutagenic potential of a number of natural products [16, 17]. Relative to the standard Ames reversion assay [18], this test system offers several advantages. For example, as opposed to the Ames system, in which each tester strain was developed to detect a specific type of mutation [18], the target in this forward mutation assay is an entire functional gene that codes for the enzyme xanthine-guanine phosphoribosyltransferase (XGPRT), which converts the selection agent 8-azaguanine to a toxic metabolite [15, 19]. Mutation is observed as colony formation in the presence of 8-azaguanine, which results from inability to metabolize 8-azaguanine due to the lack of function at XGPRT. A previous study [20] clearly demonstrated that TM677 is sensitive to several different mutagens, whereas it may be necessary to use more than one Ames strain in a single assay to obtain a response to a specific mutagen. This may not be very practical in terms of labor. In addition, there is no histidine requirement in the forward mutation assay, thus eliminating the

possibility of false-negative results when testing plant extracts which may contain appreciable amounts of histidine. Thirdly, because bacteria are plated in both the absence and presence of 8-azaguanine in the forward mutation system, a quantitative assessment of the toxicity of a test substance can be made, distinct from the mutagenicity. The Ames assay does not allow for this comparison in a direct manner.

The method described below is a modification of this forward mutation assay with *Salmonella typhimurium* strain TM677 which provides a rapid means for evaluating a large number of test substances for antimutagenic activity [10, 21].

2. Materials

A. Chemicals

1. Brain-heart infusion (BHI), No. 0037-17-8.¹
2. Sodium chloride (NaCl), No. S-5886.²
3. Potassium chloride (KCl), No. P-4504.²
4. Sodium phosphate dibasic (Na_2HPO_4), No. S-5136.²
5. Potassium phosphate monobasic (KH_2PO_4), No. P-5379.²
6. Hydrochloric acid (HCl), No. A144.³
7. Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), No. M-1880.²
8. Citric acid monohydrate, No. A104.³
9. Potassium phosphate dibasic (K_2HPO_4), No. P-3786.²
10. Sodium ammonium phosphate ($\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$), No. S-9506.²
11. Glucose, No. G-8720.²
12. Biotin, No. B-4501.²
13. Bacto agar, No. 0140-07-4.¹
14. D-glucose 6-phosphate, No. G-7879.²
15. Nicotinamide adenine dinucleotide phosphate (NADP^+), No. N-0505.²
16. Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), No. BP214.³
17. Glucose 6-phosphate dehydrogenase, No. G-7750.²
18. 8-azaguanine, No. A-8526.²
19. Dimethylsulfoxide (DMSO), No. D136-1.³
20. Tris[hydroxymethyl]aminomethane (Tris), No. T-6791.²
21. Sucrose, S-5016.²
22. Ampicillin, No. A-9518.²
23. Aroclor 1254.⁴
24. Corn oil, C-8267.²
25. 7,12-Dimethylbenz[a]anthracene (DMBA), No. D-3254.²
26. Benzo[a]pyrene, No. B-1760.²
27. Ellagic acid, No. E-2250.²

B. Glassware and plastics

1. Erlenmeyer flasks, No. 4980.⁵

2. Petri dishes, 100 mm, No. 8-757-12.³
3. Conical centrifuge tubes, 15 ml, No. 25314-15.⁵
4. Disposable pipets, 1 ml-25 ml, No. 13-678.³
5. Pipet tips, No. HR-250.⁶
6. Pipet tips, No. HR-1000.⁶
7. Pipet tips, No. RC-5000.⁶
8. Pipet tips, No. 21-197-2E.³
9. Sterile tubes, No. 14-956-6B.³
10. AcroCap 2 μm filter, No. 4480.⁷
11. VacuCap 90 bottle top 0.22 μm vacuum filter, No. 4622.⁷
12. Cotton swabs, No. 14-959-92A.³
13. Autoclave bags, No. 01-814.³
14. Syringe, No. 309580.⁸
15. Glass bottles, 125 ml, No. 02-940-5B.³
16. Glass bottles, 500 ml, No. 02-940-5D.³
17. Cryogenic vials, No. 25704-2.⁵

C. Equipment

1. Shaking incubator, No. 66799.⁹
2. Laminar flow hood, No. 92-750-4.³
3. Stirring hotplate, No. 6795 520.⁵
4. Vortex mixer, No. M37615.¹⁰
5. Jouan bench-top centrifuge, No. CR4-12.¹¹
6. Jouan high speed centrifuge, No. KR22i.¹¹
7. Polytron homogenizer, No. PT 300.¹²
8. Revco low temperature freezer, No. ULT 2090-5A.¹³
9. Manostat repetitive dispenser, No. 71-600-100.¹⁴
10. Adjustable pipet, No. P-20.⁶
11. Adjustable pipet, No. P-200.⁶
12. Adjustable pipet, No. P-1000.⁶
13. Transfer pipetter, 1-5 ml, No. 8885-890007.¹⁵
14. Adjustable pipetter, 0.5-10 μl , No. 8885-500002.¹⁵
15. Drummond pipet-aid filler/dispenser, No. 4-000-110-TC.¹⁶
16. Dry-air incubator, No. 400,¹⁷ equipped with roller drum, No. 1240.¹⁷
17. pH meter, No. 475816.⁵
18. Millipore peristaltic pump, No. XX80 200 00.¹⁸
19. Automatic colony counter, No. 003-975-0900.¹⁹

3. Procedure

A. Safety precautions

Perform all manipulations inside a laminar flow hood. Line hood with absorbent plastic-backed paper. Wear protective clothing, including disposable plastic or latex gloves at all times while performing the assay. Discard gloves immediately if contaminated with solution containing mutagens.

B. Preparation of stock solutions

1. Brain-heart infusion (BHI): Dissolve BHI

- (37 mg/ml) in distilled, deionized water. Distribute as 50 ml aliquots to glass bottles, autoclave and store at 4 °C.
2. Phosphate-buffered saline (PBS, pH 7.0): Dissolve NaCl (8.0 mg/ml), KCl (0.2 mg/ml), Na_2HPO_4 (1.15 mg/ml) and KH_2PO_4 (0.2 mg/ml) in distilled, deionized water. Adjust pH to 7.0 with HCl. Distribute to 500 ml glass bottles, autoclave and store at room temperature.
 3. Minimal essential medium: Dissolve $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 mg/ml), citric acid monohydrate (2.0 mg/ml), K_2HPO_4 (10.0 mg/ml), $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ (3.5 mg/ml), glucose (2.0 mg/ml) and biotin (final concentration 0.05 mM) in distilled, deionized water. Filter sterilize (0.22 μm) and store at 4 °C.
 4. 25X Salt solution: To 418 ml distilled, deionized water, add 2.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25 g citric acid monohydrate, 125 g K_2HPO_4 and 43.7 g $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$. Mix well. Filter sterilize (0.22 μm vacuum filter).
 5. Aqueous glucose, 20% (w/v), autoclaved.
 6. Aqueous citric acid monohydrate, 20% (w/v), filter sterilized (0.22 μm).
 7. Minimal essential bottom agar: For the preparation of 1 liter, dissolve 6 g agar in 500 ml water in an Erlenmeyer flask with heating. Autoclave the solution, permit to cool to about 60 °C, then add 100 ml 20% aqueous glucose, 40 ml of 25X salt solution, 8 ml 20% citric acid and water (autoclaved) to yield a final volume of 1 liter. Using the repetitive dispenser, distribute 15 ml aliquots of bottom agar to sterile plastic petri dishes. Permit the agar to solidify overnight.
 8. Top agar: Dissolve agar (0.6% w/v) and NaCl (0.6% w/v) in water with heating. Distribute aliquots as multiples of 90 ml (e.g., 270 ml) in glass bottles and autoclave.
 9. Cofactor solution: Dissolve glucose 6-phosphate (10.0 mg/ml), NADP^+ (10 mg/ml) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (6.7 mg/ml) in water. Filter sterilize (0.22 μm) and store as small aliquots (~3 ml) at -20 °C.
 10. Glucose 6-phosphate dehydrogenase solution: Dissolve the powdered enzyme in minimal essential media (80 units/ml) and store as small aliquots (~250 μl) at -80 °C.
 11. Biotin solution: Dissolve biotin (0.12 mg/ml) in water with extensive stirring. Filter sterilize (0.22 μm) and store at 4 °C.
 12. 8-Azaguanine solution: Dissolve 8-azaguanine (20 mg/ml) in DMSO at 37 °C. Store as 10 ml aliquots at -20 °C.
 13. Aqueous sodium chloride: 0.9% (w/v), filter sterilized (0.2 μm).
 14. 0.1 M Tris-HCl buffer: pH 7.4, filter sterilized (0.22 μm).
 15. Sucrose: 0.25 M, filter sterilized (0.22 μm).
 16. Ampicillin: 1 mg/ml in water.
 17. S9 (post-mitochondrial supernatant): Administer a single i.p. injection of Aroclor 1254 (500 mg/kg of a 200 mg/ml solution in corn oil) to male Sprague-Dawley rats (100–120 g body weight). Five days after treatment, sacrifice rats. Remove food 12 hours prior to sacrifice. Using semi-aseptic techniques, excise livers, pool and rinse several times with ice-cold sterile 0.9% NaCl. Weigh livers and mince into small pieces (> 0.5 cm). Perform the remaining steps on ice and/or at 4 °C. Homogenize livers with 3 volumes (v/w) of 0.1 M Tris-HCl buffer (pH 7.4). Centrifuge the homogenate at 800 \times g for 10 min. Discard pellet and centrifuge the supernatant for 20 min at 9,000 \times g. Remove the lipid layer using a sterile swab. Carefully decant supernatant and store as 2 ml aliquots at -80 °C. Alternatively, S9 can be obtained commercially from Microbiological Associates, Bethesda, Maryland.
 18. Stock culture: Grow *Salmonella typhimurium* strain TM677 overnight in brain-heart infusion containing 10 $\mu\text{g/ml}$ ampicillin. Add DMSO (final concentration, 10% v/v) as a cryoprotective agent and store as 1.5 ml aliquots at -80 °C.
 19. 7,12-Dimethylbenz[a]anthracene (DMBA): Dissolve DMBA (2 mg/ml) in DMSO. Store as small aliquots at -20 °C.
 20. Benzo[a]pyrene (B[a]P): Dissolve B[a]P (2 mg/ml) in DMSO. Store as small aliquots at -20 °C.
- C. Antimutagenicity assay
1. Propagation of bacteria: Distribute 50 ml BHI to a glass bottle and warm to 37 °C. Remove one vial of bacterial stock solution from storage at -80 °C, thaw rapidly and add 1 ml to the warm BHI. Incubate at 37 °C overnight (12 to 16 hours) in water bath with shaking. The following day, dilute the overnight culture 1 to 40 with minimal essential media (MEM) supplemented with 10 $\mu\text{g/ml}$ ampicillin. Incubate 3 hours at 37 °C. Dilute culture 1 to 10 in the supplemented MEM.
 2. Preparation of reaction mixture: Prepare a saturated solution (usually 10 to 50 mg/ml) of the test sample in DMSO. Distribute 10 μl in duplicate 15 ml centrifuge tubes. A known antimutagen, e.g., ellagic acid, should be included in the assay as a control antimutagen. Thaw the S9 solution quickly at 37 °C and store on ice until ready to use. Prepare reaction mixtures 'A' containing the following proportions of the indicated components:

Bacterial solution 770 μ l
 Cofactor solution 100 μ l
 S9 100 μ l
 Glucose 6-phosphate dehydrogenase 10 μ l
 Prepare reaction mixture 'B' by adding 10 μ l of the standard mutagen (in DMSO). The reaction mixtures should be added to the test compounds immediately after preparation. Add 990 μ l of reaction mixture 'B' to each centrifuge tube containing test antimutagens. Add 990 μ l of reaction mixture 'B' to duplicate tubes containing 10 μ l DMSO. Add 980 μ l of reaction mixture 'A' to duplicate centrifuge tubes containing 20 μ l DMSO. Solution 'A' may also be used to assess the mutagenic potential of the test antimutagens. Place tubes on a roller rack and incubate the reaction mixtures for 2 hours at 37 °C. A typical protocol follows:

Tube Sample

1	DMSO	
2	DMBA (20 μ g/ml)	
3	B[a]P (20 μ g/ml)	
4	Compound A (100 μ g/ml)] mutation test
5	Compound A (20 μ g/ml)	
6	Compound A (4 μ g/ml)	
7	Compound A (0.8 μ g/ml)	
8	Compound A (0.16 μ g/ml)	
9	Ellagic acid (100 μ M)] + DMBA
10	Compound A (100 μ g/ml)	
11	Compound A (20 μ g/ml)	
12	Compound A (4 μ g/ml)	
13	Compound A (0.8 μ g/ml)	
14	Compound A (0.16 μ g/ml)	
15	Ellagic acid (100 μ M)] + B[a]P
16	Compound A (100 μ g/ml)	
17	Compound A (20 μ g/ml)	
18	Compound A (4 μ g/ml)	
19	Compound A (0.8 μ g/ml)	
20	Compound A (0.16 μ g/ml)	

Each tube indicated above is prepared in duplicate. The solutions required for this assay are:

- 4000 ml bottom agar (240 plates \times 15 ml = 3600 ml agar)
- 900 ml top agar (450 ml each 'toxicity' and 'mutation agar')
- 100 ml biotin solution
- 10 ml 8-azaguanine solution
- 14.85 ml bacterial solution 'B' with DMBA (rounded up to 15 samples)
- 14.85 ml bacterial solution 'B' with B[a]P (rounded up to 15 samples)
- 14.85 ml bacterial solution 'A' (rounded up to 15 samples)

3. Preparation for plating: During the incubation period, heat an adequate number of bottles containing top agar until boiling. Supplement the liquid agar with 10 ml sterile biotin solution (prewarmed to 45 °C) per 90 ml top agar. This will be referred to as 'toxicity' agar. Supplement one-half of the toxicity agar with 2 ml 8-azaguanine solution (prewarmed to 45 °C) per 90 ml agar. It is important to add the 8-azaguanine while the liquefied agar is still near the boiling point. The agar supplemented with 8-azaguanine will be referred to as 'mutation' agar. For each experimental point, prepare a vial containing 10 ml toxicity agar, a vial containing 10 ml mutation agar and a vial containing 4 ml PBS. Place the vials containing agar in a 45 °C water bath. Place the vials containing PBS in the laminar flow hood. For each experimental point, label 3 petri dishes containing bottom agar for toxicity agar and 3 for mutation agar.
4. Preparation of bacteria for plating: After the 2 hour incubation, add 4 ml PBS to each centrifuge tube. Vortex briefly and centrifuge the tubes at 1,000 \times g for 15 min. Decant the supernatant into a sterile waste container. Add 5 ml PBS to each pellet. Vortex *thoroughly* to resuspend bacteria.
5. Plating and incubation: Pipet 150 μ l of the resuspended bacteria to a vial containing mutation agar. Vortex. Using a new pipet tip, add 150 μ l of this to a vial containing PBS. Vortex. Using a new pipet tip, add 150 μ l of this to a vial containing toxicity agar. Using a pipet, layer 2.5 ml aliquots of the toxicity agar containing bacteria over each of 3 petri dishes containing bottom agar. Without changing the pipet tip, layer 2.5 aliquots of the mutation agar containing bacteria over each of 3 petri dishes containing bottom agar. Allow the top agar to solidify for several minutes. Invert the plates and incubate at 37 °C for 48 hours.
6. Enumeration of colonies: Using the automatic colony counter, quantify the colonies on each plate.
- D. Analysis of data. Average the number of colonies observed on the mutation (M) and toxicity (T) plates and, when the described dilution scheme is used, apply the following formula to calculate the mutant fraction (mf), the ratio of mutants per 10⁵ survivors:

$$mf = [(M/T) \times (0.150/4.15) \times (0.150/10.150) \times 10^5],$$

which simplifies to

$$mf = (M/T \times 53).$$

Calculate percent inhibition according to the following formula:

$$\% \text{ Inhibition} = [1 - (\text{mf}_{\text{test sample}} - \text{mf}_{\text{DMSO}}) / (\text{mf}_{\text{mutagen}} - \text{mf}_{\text{DMSO}})] \times 100$$

4. Results and discussion

This paper describes a method for detecting inhibitors of forward mutation induced by DMBA and B[a]P. These two mutagens are used in a number of systems in our laboratory, including DNA-binding and in vivo carcinogenesis studies. Thus, some comparison of results can be made among the different assays. A variety of other mutagens, both indirect-acting (requires S9 fraction for activation to the ultimate mutagen) and direct acting (interacts directly with DNA; does not require S9) can be used in this system [20].

The enzymatic activity of the S9 fraction can vary from batch to batch, so it is important to perform dose-response studies to optimize the amount needed in the reaction mixture to produce the desired activity. Too much, as well as too little, S9 can inhibit the mutagenic response [19]. In our experience, 5% S9 is optimal for DMBA activation, while 10% S9 is added for B[a]P activation. When DMBA is used in the assay, the remaining 50 μ l in each reaction mixture can be substituted with MEM or additional bacteria. In our laboratory, S9 preparations were stable for at least 2 years when stored at -80°C . Other laboratories have reported the same findings [18]. To maintain consistency of results, excess S9 should be discarded and not reused.

Under the conditions described, the maximum number of colonies per plate should be between 200 and 400. This permits accurate quantification with the colony counter. The mutant fractions will vary somewhat from assay to assay, but the spontaneous (DMSO-treated) mutant fraction should always be less than 10. The mutant fractions induced by DMBA and B[a]P should always be at least 3 times higher than the spontaneous mutant fraction. In addition, although the mutant fractions may vary, the inhibitory activity of an active antimutagen should remain consistent from assay to assay. If the mutagenic response of the bacteria seems consistently low, i.e., is $< 3\times$ the mutant fraction of the DMSO-treated control in repeated assays, or if the spontaneous mutants are consistently high (> 10), the strain should be regenerated. In order to regenerate bacteria, plate a diluted culture onto minimum agar plates. Select several single colonies and grow each overnight in 5 ml BHI as described above. Following the same protocol as above, determine the mutagenic response of each colony. Select a colony which gives a desirable profile, i.e., low spontaneous mutants and high sensitivity to the mutagen and prepare as a stock culture.

Table 1 lists compounds which have demonstrated antimutagenic activity in the forward mutation assay. The compounds diallyl sulfide and limonene were extremely toxic to the bacteria; however, the antimutagenic activity can be distinguished from the bactericidal effect. Dose-response data for ellagic acid, which is routinely included in assays as a positive control mutagen, are shown in Figure 1. Over 40 plant extracts have been tested for antimutagenic activity in our laboratory. A number of them are currently undergoing bioassay-directed fractionation for isolation of the active antimutagens.

Table 1. Inhibitory effects of potential cancer chemopreventive compounds on DMBA-induced mutations in *Salmonella typhimurium* strain TM677

Compound	% Inhibition*	% Survival
N-Acetyl-L-cysteine	64	100
Apigenin	67	98
Brassinin	65	94
β -Carotene	51	100
Curcumin	55	100
Dehydroepiandrosterone	78	100
Diallyl sulfide	73	21
Ellagic acid	46	100
Limonene	89	2
Nordihydroguaiaretic acid	83	100
Quercetin	71	84
Retinoic acid	46	100
Sodium selenite	100	82

* % Inhibition at 100 $\mu\text{g/ml}$.

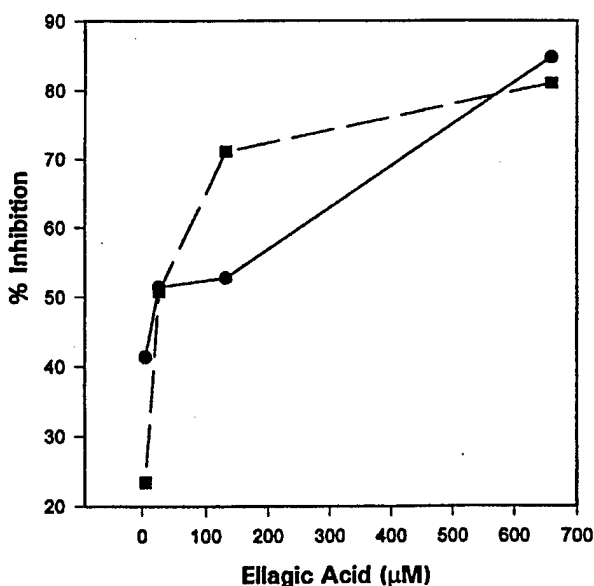


Figure 1. Dose-dependent inhibition of the mutant fraction induced by DMBA (●) or B[a]P (■) by ellagic acid.

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Notes on suppliers

1. Difco Laboratories Inc., Detroit, MI, USA
2. Sigma Chemical Co., St Louis, MO, USA
3. Fisher Scientific Corp., Itasca, IL, USA
4. Ultra Scientific, North Kingstown, RI, USA
5. Corning Inc., Corning, NY, USA
6. Rainin Instrument Co. Inc., Woburn, MA, USA
7. Gelman Sciences Inc., Ann Arbor, MI, USA
8. Becton Dickinson, San Jose, CA, USA
9. Precision Scientific Inc., Chicago, IL, USA
10. Barnstead/Thermolyne Corp., Dubuque, IA, USA
11. Jouan Inc., Winchester, VA, USA
12. Brinkman Instruments Inc., Westbury, NY, USA
13. Revco Scientific Inc., Asheville, NC, USA
14. Manostat Corp., New York, NY, USA
15. Oxford Labware, St. Louis, MO, USA
16. Drummond Scientific Co., Broomall, PA, USA
17. Lab-Line Instruments Inc., Melrose Park, IL, USA
18. Millipore Corporation, Bedford, MA, USA
19. Imaging Products International Inc., Chantilly, VA, USA

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Evaluation of the mutagenic, cytotoxic, and antitumor potential of triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii*

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Abstract

Triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii* Hook f. (Celastraceae), has been shown to demonstrate potent antileukemic activity in rodent models at remarkably low treatment doses. A variety of other physiological responses are known to be mediated by this compound, including immunosuppressive and antifertility effects. We currently report that triptolide was not mutagenic toward *Salmonella typhimurium* strain TM677, either in the presence or absence of a metabolic activating system. Relatively potent but non-specific cytotoxicity was observed with a panel of cultured mammalian cell lines, and modest antitumor activity was observed when an i.p. dose of 25 μ g was administered three times weekly to athymic mice carrying human breast tumors. Treatment regimens involving higher doses of triptolide (e.g. 50 μ g/mouse three times weekly) were lethal. © 1997 Elsevier Science Ireland Ltd. All rights reserved

Keywords: Triptolide; Bacterial mutagenesis; Cytotoxicity; Antitumor activity

1. Introduction

Triptolide, a highly oxygenated diterpene (Fig. 1), was first isolated by Kupchan et al. from an ethanol extract of the Chinese medicinal plant *Tripterygium*

wilfordii Hook f. (Celastraceae) on the basis of bioassay-directed fractionation [1]. This compound demonstrated impressive in vivo activity against murine L-1210 leukemia with doses in the range of 50–400 μ g/kg body weight [2]. In other studies, dosages of 200 and 250 μ g/kg prolonged the survival time of mice bearing L615 leukemia [3]. Also, intense cytotoxicity with cultured KB cells was observed, with ED₅₀ values in the range of 0.1–1.0 ng/ml [2,4]. All of

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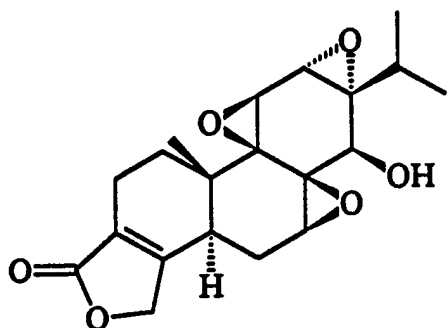


Fig. 1. Structure of triptolide.

these data suggest triptolide may be useful as an anti-tumor agent. However, studies have not been reported describing the efficacy of triptolide using more contemporary models of cancer, such as athymic mice bearing solid human tumors.

In other areas of therapeutic relevance, strong interest has developed in the use of *T. wilfordii* as an immunosuppressive agent [5] and an antifertility agent [6], and it has been reported that many biological effects of *T. wilfordii* can be attributed at least in part to triptolide [7–9]. However, the plant is known to be highly poisonous and severe toxicities and deaths have been attributed to exposure [10,11].

Due to the potential use of triptolide in therapeutic settings, or the possibility of exposure by other intentional or accidental circumstances, it was of interest to assess mutagenicity with *Salmonella typhimurium* strain TM677. In addition, we currently report the potential of triptolide to mediate cytotoxic activity with a panel of cultured mammalian cell lines, and antitumor effects with athymic mice carrying human breast tumors.

2. Materials and methods

2.1. Isolation of triptolide

The crude extract used in this investigation was prepared by Taizhou Pharmaceutical Factory, Taizhou, Jiangsu, People's Republic of China, from the alcoholic extract of the root xylem of *Tripterygium wilfordii* Hook f. (Celastraceae). The brownish powder obtained was directly used for the isolation of triptolide. The crude extract (400 g) was filtered

over neutral alumina by CC, eluting with a CHCl_3 –MeOH gradient of increasing polarity. Fractions were combined on the basis of TLC and the violet color observed with Kedde reagent. Fraction 3 (106.2 g, intense violet color with Kedde reagent), eluted with CHCl_3 /MeOH (33:1 to 25:1), was freeze-dried and precipitated with MeOH. The solid (10.2 g; preliminary NMR data indicated a mixture of alkaloids reported from *T. wilfordii* [12]) was filtered and the residue (95.8 g) obtained from evaporation of the mother liquor was subjected to flash CC [13] over Si gel (E. Merck no. 7736), eluting with a hexane–acetone gradient of increasing polarity. Fractions were combined on the basis of TLC (sprayed with vanillin) and Kedde reagent. Fraction 9 (9.75 g, deep violet color with Kedde reagent), which was eluted with hexane/acetone (5:1 to 4:1), gave triptolide as white powder (0.50 g) on crystallization with ether. The residue (9.25 g) from the mother liquor was further purified with MPLC [13] (Büchi B-680, Flawil, Switzerland) using Si gel (E. Merck no. 60, 40–63 μm , column size 7.0 \times 23 cm); flow rate 50 ml/min; combined isocratic and gradient elution, consisting of seven consecutive segments (quoted as % in MeOH in CH_2Cl_2): 0–0 (60 min), 0–2 (60 min), 2–2 (60 min), 3–3 (60 min), 3–5 (60 min), 5–10 (60 min) and 10–100 (30 min). Fraction 2 (from segments 0–2 to 3–3) gave triptolide (0.11 g) as white powder on crystallization with ether. Recrystallization of the combined white powder gave triptolide (0.60 g, >99% purity) as white needles (CH_2Cl_2 – Et_2O): mp 236°C; $[\alpha]_D^{160}$ (C 0.364, CH_2Cl_2); spectroscopic data were identical to those reported [1] and to an authentic sample.

2.2. Evaluation of mutagenic activity

The mutagenic potential of triptolide was analyzed at various concentrations with *Salmonella typhimurium* strain TM677 as previously described [14,15]. Briefly, bacteria grown in minimal essential medium were incubated with various concentrations of triptolide (dissolved in DMSO) in duplicate in the presence and absence of a metabolic activating system (Aroclor 1254-induced post-mitochondrial supernatant derived from rat liver). Following incubation, bacteria were cultured with or without 8-azaguanine on minimal essential agar plates. Colonies were quantified after

Table 1

Cytotoxicity of triptolide mediated with cultured mammalian cell lines

Cell line ^a	ED ₅₀ (ng/ml)
UIISO-BCA-1	6
UIISO-BCA-2	20
SK-BR-3	10
ZR-75-1	10
HBL-100	1
LNCaP	10
UIISO-LUC-1	10
KB-3	20
KB-V1	10
P-388	1

^aOrigin of cell lines: UIISO-BCA-1, UIISO-BCA-2, SK-BR-3, ZR-75-1, human breast carcinomas; HBL-100, immortalized human breast cells; LNCaP, human prostate carcinoma; UIISO-LUC-1, human lung carcinoma; KB-3, human oral carcinoma; KB-V1, vinblastine-resistant KB cells; P-388, murine leukemia.

a 40 h incubation at 37°C and the mutant fraction was calculated as a ratio of colonies on plates containing 8-azaguanine to colonies on plates with no 8-azaguanine.

2.3. Evaluation of cytotoxic activity

UIISO-BCA-1 [16], UIISO-BCA-2 [16] and UIISO-LUC-1 cell lines were established from primary tumors in the Department of Surgical Oncology, University of Illinois at Chicago. SK-BR-3, ZR-75-1, HBL-100, LNCaP and P-388 cell lines were purchased from the American Type Culture Collection (Rockville, MD). KB-3 and KB-V1 [17] cell lines were provided by Dr. I.B. Roninson (Department of Genetics, University of Illinois at Chicago). Cells were maintained according to supplier recommendations or as previously described [18].

The cytotoxicity of triptolide was determined with the above cell lines according to the sulforhodamine B (SRB) protocol described previously [18]. Cells in logarithmic growth were harvested by trypsinization (with the exception of P-388 cells), quantified, diluted in media and distributed to 96-well plates containing various concentrations of triptolide (dissolved in DMSO) in triplicate; the final DMSO concentration was 0.05%. The plates were incubated for 72 h, after which the cells were fixed with trichloroacetic acid and stained with 0.4% SRB in 1% acetic acid. The

bound dye was solubilized with 0.1 M Tris base and the absorbance A_{515nm} was measured with a microtiter plate reader. The growth of triptolide-treated cells was compared with growth of DMSO-treated controls, the results were expressed as a percentage and ED₅₀ values were generated from dose-response curves.

2.4. Animals and treatments

Female Balb/c athymic mice, 4–6 weeks old (Frederick Cancer Facility, Bethesda, MD) were weighed and separated into groups of 4–6 mice each. UIISO-BCA-1 cells (1×10^6 cells/animal) were injected subcutaneously in the dorsal flank region. Control animals received 10% DMSO in saline solution (intraperitoneal injection, i.p.) while treated animals received 5–50 µg triptolide/mouse (i.p.) in 10% DMSO/saline. Drug treatment was initiated 72 h after tumor inoculation and continued at regular intervals for a total of ten doses. Animals were weighed and tumor volume was determined using vernier calipers throughout the course of the experiment.

3. Results and discussion

When tested at concentrations ranging up to 800 µg/ml, triptolide did not demonstrate activity in a forward mutation assay with *Salmonella typhimurium* strain TM677, either in the absence or presence of a metabolic activating system (data not shown). Even though the α,β -unsaturated carbonyl and the epoxide functionalities are each known to induce mutagenic effects [19–21], and both groups are present in the triptolide molecule, the lack of mutagenic potential suggests that triptolide does not covalently interact with DNA. These results agree with an earlier study [22] in which triptolide was found to inhibit DNA synthesis in L-1210 leukemia cells without directly damaging DNA.

Triptolide demonstrated potent cytotoxicity with a panel of cultured mammalian cell lines (Table 1). ED₅₀ values ranged from 1 to 20 ng/ml. This cytotoxic response profile is consistent with activity profiles observed with various cancer chemotherapeutic drugs, such as camptothecin, etoposide, taxol, etc., which demonstrate relatively intense activity (ED₅₀ values ranging from 10^{-4} to 10^0 µg/ml) with no discernible cell-type specificity [23]. In addition, tripto-

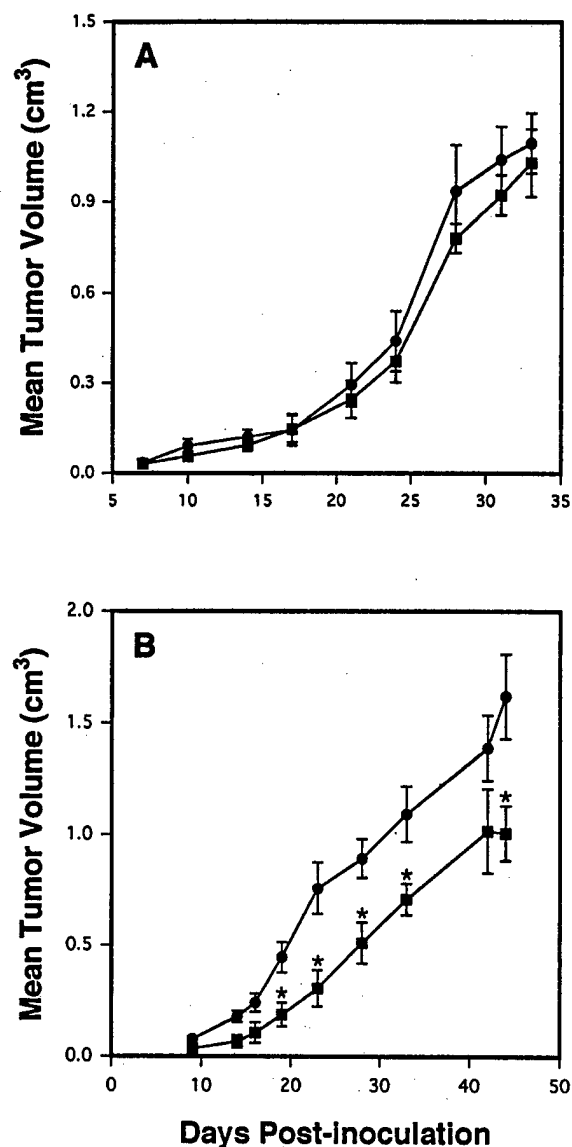


Fig. 2. Evaluation of antitumor activity of triptolide against human breast cancer cells inoculated into athymic mice. Triptolide (■) or DMSO (●) was administered i.p. three times weekly. (A) Five µg/mouse, five animals/group; (B) 25 µg/mouse, four animals/group. Data is expressed as mean tumor volume \pm standard error. No statistically significant ($P < 0.05$) antitumor activity was observed in the treatment group receiving 5 µg/mouse. An * indicates statistical significance at $P < 0.05$. Procedural details for in vivo studies are given in Section 2.

lide has been reported to inhibit colony formation of several human cancer cell lines [24]. Therefore, we tested triptolide for antitumor activity in athymic mice

carrying the human breast cancer cell line UIISO-BCA-1. Dosage was 5 µg/mouse (approximately 250 µg/kg body weight) three times a week, which corresponds to the mid-range dose used in the studies by Kupchan and coworkers [1]. As shown in Fig. 2A, this regimen did not affect tumor growth in athymic mice. Therefore, the experiment was repeated, and the dose of triptolide was increased ten-fold to 50 µg/mouse. Within 3 days of the first injection of this dose, 50% of the treated mice expired. With the surviving animals, treatment with triptolide continued, but the dose of subsequent injections was reduced to 25 µg/mouse. As illustrated in Fig. 2B, this dose was effective in reducing tumor growth, relative to controls. Similar to the group receiving 5 µg/mouse, no overt signs of toxicity, such as decrease in body weight, were observed in the test group given 25 µg/mouse (data not shown).

It can be concluded that while triptolide is capable of demonstrating some antitumor activity with this model system, it is certainly not curative, and the range of efficacy is narrow. The agent may represent a suitable lead for combinatorial studies in which therapeutic efficacy may be enhanced.

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11. Appendix 2
Animal Use Report

Animal Use Reporting

Breast Cancer Research Training Program
University of Illinois at Chicago
Board of Trustees
809 South Marshfield Ave., M/C 551
Chicago, IL 60612-72095

Activity Name & Address Contract Number DAMD17-94-J-4446

Animal Type Genus/Species	Animals Purchased or Bred	Animals Used	USDA Pain Column C	USDA Pain Column D	USDA Pain Column E	AAALAC Accreditation
Nude Mice		70		70		
C57 black MUC1-knockout	10 bred to 50	50		50		
C57 black MUC1- transgenic	6 bred to 100	100		100		
C3H mouse tumor virus (MTV)-positive		10		10		
Balb/C		110		110		

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